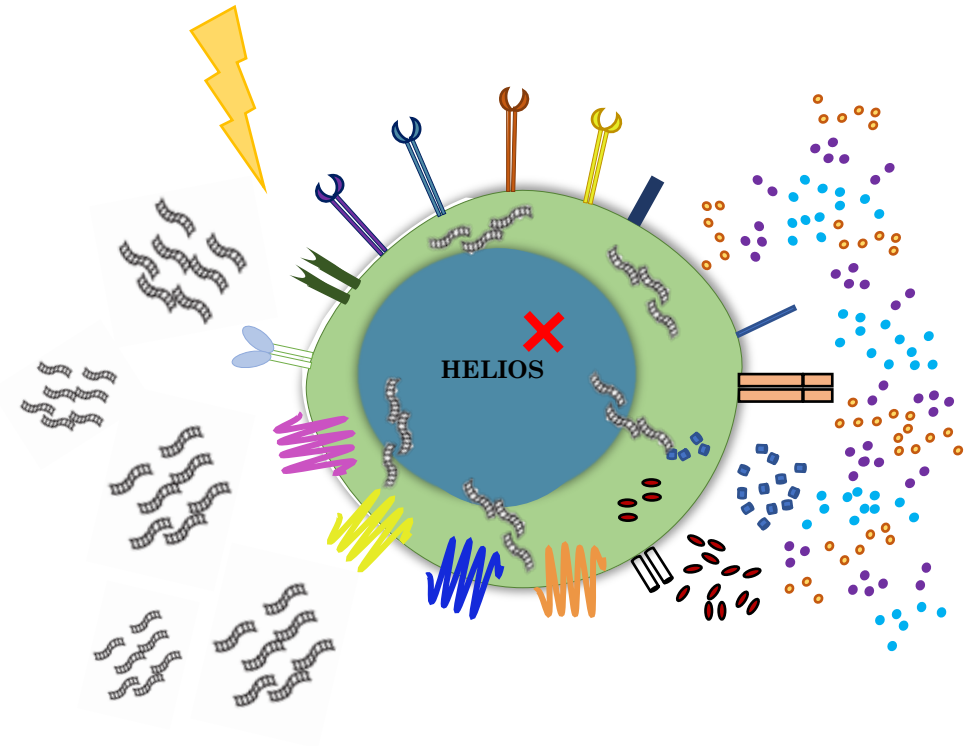
Exploring the Functional Role of Helios in Mucosal Associated Invariant T Cells using siRNA.



UNIVERSITY OF HELSINKI

Kirsten Helene Anna-Marie Nowlan

Master's Thesis

International Master's Degree Programme in Translational Medicine

Faculty of Medicine

University of Helsinki

2020

ABSTRACT

Mucosal associated invariant T (MAIT) cells are a fairly recently described population of innate-like T cells. In humans, MAIT cells represent an exceptionally abundant population in the blood, where they account for 1–10% of all T cells. However, compared to conventional T cells, which can display an almost unlimited T cell receptor (TCR) repertoire, the specificities of MAIT cell TCRs are limited. This evolutionarily conserved subset displays a semi-invariant TCR which recognises riboflavin metabolites, produced by a wide range of bacteria and fungi, and presented on the major histocompatibility complex (MHC)-class I related (MR1) molecule. The function and significance of MAIT cells in health and disease have only started to be unravelled, and it is becoming increasingly clear that MAIT cells are also modulated in non-microbial diseases. Interestingly, MAIT cells have been shown to exhibit a relatively high expression of the transcription factor, Helios, compared to most other T cell subsets. The function of this translational activator and repressor, encoded by the gene IKZF2, in the MAIT cell population remains obscure. This study focused on the functional role Helios may play in regulating the activation of MAIT cells. Thus, by using siRNA to silence Helios expression, and flow cytometry to analyse any potential alterations in MAIT cell activation markers, we aimed to be able to characterise the kinetics and functional role of Helios in peripheral MAIT cells of healthy individuals. Here, we clearly established a striking upregulation of Helios in MAIT cells following 24 hours of stimulation. Moreover, we were able to achieve a >50% knockdown of Helios at the protein level, in this subset of T cells. Nevertheless, no significant difference in any of the activation markers we investigated was present between the MAIT cells with reduced Helios expression and their controls. This could, however, of occurred as a result of the toxicity that the transfection had on the functionally of the T cells. From these results, it is difficult to conclude any clear role for Helios in the activation of MAIT cells, and consequently, further research needs to be performed before any clear conclusions can be drawn.

LIST OF ABBRIVIATIONS

ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APC	Antigen-presenting cell
BSA	Bovine serum albumin
CCR	CC chemokine receptor
CD	Cluster of differentiation
$CD4^+$	$CD8^-$ $CD4^+$ cell
$CD8^+$	$CD8^+$ $CD4^-$ cell
CXCR	CX chemokine receptor
DAMPs	Damage associated molecular patterns
DC	Dendritic cell
DCM	Dead cell marker
DN	Double negative, $CD8^-$ $CD4^-$ cell
DP	Double positive, $CD8^+$ $CD4^+$ cell
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescent-activated cell sorting
FBS	Foetal bovine serum
Grz	Granzyme
HCV	Hepatitis C virus
HIV	Human immunodeficiency viruses
INF	Interferon
IKZF2	IKAROS family zinc finger 2 *(also known as Helios)*
IL	Interleukin
JAK	Janus kinase
KD	Knockdown
µ	Micro (10^6)
mAbs	Monoclonal antibodies

MACS	Magnetic-activated cell sorting
MAIT cell	Mucosal associated invariant T cell
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MR1	Major histocompatibility complex class-I related
NK cell	Natural killer cell
NKT	Natural killer T cell
NURD	Nucleosome remodelling and deacetylase
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PLZF	Promyelocytic leukaemia zinc finger
pmol	Picomole
PMTs	Photomultiplier tubes
Prf	Perforin
RORyt	Retinoid-related orphan receptor yt
rpm	Revolutions per minute
RT	Room temperature
siRNA	Small interfering ribonucleic acid
STAT	Signal Transducer Activator of Transcription
Tbet	T-box binding transcription factor
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	T regulatory
5-OE-RU	5-(2-oxoethylideneamino)-6-D-ribitylaminouracil
5-OP-RU	5-(-2-oxopropylideneamino)-6-D-ribitylaminouracil
6-FP	6-formylpterin

TABLE OF CONTENT

1 INTRODUCTION

In a world so abundantly populated with pathogens, the human body has developed multidimensional protective mechanisms that together constitute the immune system. This amazingly complex system can recognise, and hence protect from, millions of pathogenic microbes that individuals may have never before encountered.

However, like any system, the immune system has the ability to malfunction, which leads to the development of autoimmune diseases allergy and even immunodeficiencies. T cells play a significant role in the maintenance of tolerance in health, but also when immune dysregulation occurs, they have a direct role in the development of tissue damage, which encompasses autoimmune diseases. Consequently, T cells should be viewed as a profoundly important target for pathologies associated with the disturbances of the immune system. Thus, gaining a more comprehensive understanding of T cell immunology will contribute to the development of new therapies that can treat the cause of these diseases. Moreover, the potent effector phenotype that T cells display have the potential to be utilised and specifically augmented against targets for treatments of other conditions such as cancers.

Ultimately, a deeper understanding of T cell differentiation, diversity, dynamics, repertoires selection, and regulation processes is critical for the advancement of fundamental research, and thus, for the innovation of new medical treatments and drug discovery.

Here, we aim to provide further knowledge on the function and regulation of a subset of T cells known as mucosal-associated invariant T cells, by exploring the role of the transcription factor Helios in the activation of these T cells.

2 LITERATURE REVIEW

2.1 The Immune System

The immune system is a continually evolving network designed to protect us from a world heavily populated with pathogenic microbes. It is characterised not only by its vast network of inter- and intra-cellular signalling but also by its exceptional lymphocyte receptor diversity, its capacity to migrate throughout the body, maintain homeostatic regulation, and rapidly adapt to a changing environment[1,2,3]. To simplify this complexity, the immune system is often described as having two fundamental lines of defence: the innate and adaptive immune response, in which both mechanisms are utilised harmoniously to discriminate and eliminate dangers[1,3].

2.1.1 Innate Immunity

Innate immunity represents the first line of defence to an intruding pathogen. It comprises of anatomical, physical and inflammatory barriers[4]. One critical defence mechanism that is utilised by this system is the ability to recognise pathogen-associated molecular patterns (PAMPs), which are highly conserved structural motifs common to many pathogenic microbes and toxins[5,6]. This type of hard-wired response is encoded in the germline of the host, and upon activation, it incites the recruitment of more immune cells to sites of infection through the production of cytokines and chemokines[3,7]. Furthermore, innate immune cells are also able to perceive immunological 'danger' in the form of damage-associated molecular patterns, which characterise the common metabolic effects of infection and inflammation[5,7]. These are molecules that are upregulated and released during the cell lysis and tissue damage processes in the context of both infectious and sterile inflammation[3,5]. Additionally, innate immune receptors also detect the lack of specific molecules, typically expressed by normal, healthy cells but are not present on cells infected by microbial pathogens[3,5,8]. This occurs by way of the absence of an inhibitory

signal that would normally be delivered to prevent activation of the immune response against host tissues[3,9]. The broad ranging exploitation of these recognition molecules across a large number of cells within the innate immune system, such as dendritic cells, macrophages, neutrophils, mast cells, basophils, eosinophils, natural killer (NK) cells and innate lymphoid cells, is the basis for why the system is poised to act rapidly[10]. Speed is a defining feature of the innate immunity. Within minutes of exposure to a pathogen the innate immune system generates a protective inflammatory response, and hence constitutes the initial host response[3,11]. Soluble proteins also facilitate this rapid response for instance; complement proteins that are constitutively present in biological fluids, and small bioactive molecules like cytokines that regulate the function of other cells, or chemokines that attract inflammatory leukocytes, and lipid mediators of inflammation[1,3]. While innate immunity is essential for defence against pathogenic challenges, it is also emerging as a key regulator of human inflammatory disease[12,13]. Additionally, innate immunity plays a central role in initiating the subsequent adaptive immune response, and in order to avoid damage to self-tissues, it is essential that the entirety of immune response is able to avoid the destructive mechanisms against the mammalian host's own tissues.

2.1.2 Adaptive Immunity

The adaptive immune system has evolved into a system of billions of highly diversified lymphocytes all interacting as a connective dynamic multi-scale network, to ensure a sophisticated immune response. The response of the adaptive immune system is triggered when the innate immune response is insufficient to control an infection[14]. Synergy between these two arms is fundamental for an intact, fully effective immune response[3]. The overwhelming variability of antigenic structures, as well as the ability of pathogens to mutate to avoid host detection has driven the evolution of the adaptive immune system. The adaptive response primarily involves the expression of antigen-specific receptors on the surfaces of T- and B- lymphocytes and a tightly

regulated interplay between antigen presenting cells (APCs)[15]. During lymphocyte development, these antigen-specific receptors are selected and precisely tailored by a process of somatic recombination, ensuring that their diverse repertoire bind appropriately to the corresponding antigen and are not self-reactive[16,17,18]. Upon recognition of their corresponding antigen, lymphocytes undergo clonal expansion, which facilitates pathogen-specific immunological effector functions[17]. Effector pathways of the adaptive system are classically described as either humoral or cell mediated. The humoral immune system is typically associated with the functions of B lymphocytes, and targets antigens from pathogens that are freely circulating, or displayed on the exterior of infected cells[19]. Cellular immunity, however, directs it effector mechanisms towards intracellularly infected cells and is mediated by T lymphocytes[20]. Following a response to a particular pathogen, the adaptive immune system also evolves a memory to provide the host with long-term protection from reinfection from the same pathogen and on re-exposure; this memory facilitates faster and more effective response[21,22].

2.1.3 T Cells and Their Function in the Immune System

T lymphocytes are a fundamental part of the adaptive immune system. Their roles encompass the direct elimination of infected host cells, activation of other immune cell populations, the production of cytokines and regulation of the immune response[23]. These key features of T cell responsiveness are established during their development in the thymus, upon the production of their characteristic surface receptors, known as T cell receptors (TCRs) which essentially programme their capacity to protect the body against a diverse range of pathogens, while maintaining tolerance to self-antigens[16,17,18,24]. TCRs are expressed at the cell surface as a complex, which contains signalling chains TCRζ and CD3 complex that generate intracellular signals upon antigen recognition. The heterodimeric TCRs consist of two distinct polypeptide chains, linked by a disulphide bond. Each T cell expresses monospecific

TCR complexes where all receptor complexes in a given cell harbours the same uniquely rearranged TCR**α** and TCR**β** sequence, which is typically specific to a single antigen[25]. The diversity that is required to generate unique TCR complexes for each T cell is vast and commands a complex mechanism involving the rearrangement of the germline TCR genes[17,18,26]. Each TCR locus contains numerous copies of DNA segments which encode the variable domain of each TCR chain, and are denoted as variable (V), Joining (J) and, for the TCR**β** and TCR**δ** chain, Diversity (D). Such copies differ in their sequence and are arranged to include a single copy of each V, D and J segment[17]. Moreover, further diversification results from nucleotide deletions and non-templated additions that are produced during the joining process[18]. The structural formation of the **α** and **β** chains resulting from the rearrangement have three hypervariable complementarity determining regions, the third of which has the most polymorphism and hence has the primary role in distinguishing the peptide[26]. The diversity of TCR repertoire that this process produces primes T cells for the recognition of a whole spectrum of antigens, however the ability of T cells to recognise these antigens typically requires their presentation by other immune populations known as, antigen-presenting cells (APCs), hence partially restricting the capacity of T cells to activate their effector functions[27,28].

2.1.4 Antigen Presentation and T Cell Activation

In the presence of infection or inflammation, APCs such as dendritic, macrophage and also B cells, present antigens on the "classical" major histocompatibility complex molecules, class I (MHC-I) or class II (MHC-II), that have the potential to activate T cells[17,29]. Antigen recognition occurs through the interaction between the two TCR chains with both the corresponding peptide antigen and MHC amino acid residues. Upon identification of a specific MHC-peptide complex, such T cells proliferate and acquire properties of a mature T cell, such as cytokine production or expression of specific surface molecules[18]. Whether T cells recognise MHC I or MHC II depends on

their co-receptor expression, which is determined during T cell development in the thymus[30]. These two surface glycoproteins contribute to the recognition of MHC molecules and signalling by T cells. $CD8^+$ T cells identify antigen peptides bound to the MHC I molecules and $CD4^+$ T cells peptides bound to MHC II molecules[31,32]. Both $CD4^+$ and $CD8^+$ subsets have distinct functional roles within the immune system. Naive CD4+T cells are activated after interaction with antigen-MHC II complex on APCs and differentiate into discrete effector subtypes, depending largely on the cytokine milieu of the microenvironment[33]. Collectively, $CD4^+$ T cells are often referred to as helper T cells (Th) as they are chiefly responsible for the activation of other cells within the immune system such as B-lymphocytes and $CD8^+$ T cells. However, some subtypes also play a vital role in the suppression of immune reaction (regulatory T cells, Tregs) [18]. $CD8^+$, on the other hand, recognises MHC I, which are expressed by all the nucleated cells and primarily presents peptides derived from intracellular degradation of proteins. The pathogen-derived antigen presented in the MHC I molecule indicates an intracellular infection and directs cytotoxic $CD8^+$ T cells to kill the infected cell[34].

The immune system is exceptionally potent, particularly the T cell response. Evidently this response has the clear potential to exhibit destructive effects on one's own tissues when mediation is impaired[35]. Therefore, it requires tight regulation. Three signals are required for conventional T cell activation: TCR-MHC: peptide-interaction, a co-stimulatory signal that is provided by CD28 and CD80/CD86, and cytokines[36]. Crucially, the third signal, derived from the cytokine environment, drives $CD4^+$ T helper cell subset differentiation[37].

2.1.5 Th Responses

T lymphocytes depend upon the function of immunological networks that initiates, guides and controls them. The specific binding of cytokines with their receptors on helper T cells results in the stimulation of JAK/STAT pathways, and ultimately leads

to the augmented expression of subset-specific genes, including those that encode "lineage-defining" transcription factors[38,39]. Immune reactions *in vivo* constitute several different Th response types, and it is the balance between these that determine the immunological outcomes. The influential research conducted by Mosmann and Coffman in 1986 found that naïve $CD4^+$ T cells were able to differentiate into two distinct subsets, either T helper 1 (Th1) or Th2[40]. This discovery launched a new field of immunological investigation designed to understand the mechanisms that underlie the functional diversity of $CD4^+$ subsets. For a long period, many thought that only the original Th1 and Th2 subsets existed. However, more recent research has shown that T helper cells are not only limited to these two subsets but have been expanded to include the subpopulations now classified as Th17, follicular helper T cells (Tfh), and induced Tregs[41,42,43]. As evident by these described populations, $CD4^+$ T helper cells are capable of differentiating into a number of effector subsets that each provide discrete functions during an adaptive immune response (***Figure 1***). Environmental cytokine signals largely govern the differentiation of each of these subsets and the subsequent activation of downstream transcription factor pathways. However, plasticity between these response types have also been observed[44]. The diverse functions executed by these subpopulations permit an extremely tailored pathogen-specific immune response to bacterial, viral, and parasitic infections.

The Th1 cells are critical in the response against intracellular bacteria, fungi and protozoa[40]. The secretion of IFN-**γ**, and lymphotoxin-**α** are characteristic of this subset, and results in the enhanced antimicrobial activity of macrophages and DCs, thus, augmenting their ability to eradicate intracellular microbes and their ability to present antigens[45]. Moreover, this response is aided through the direct interaction of CD40-L with CD40R on the surface of APCs[46]. Naïve $CD4^+$ T cells are induced into the Th1 lineage by early exposure to several cytokines, but the two most critical at the time of T cell priming are IFN-**γ** and IL-12, with the initial sources of these signals

thought to derive from NK cells and DCs, respectively[45]. Through the activation of the STAT 1 pathway, IFN-**γ** induces the expression of T-bet, a Tbox transcriptional regulator, that in turn promotes the upregulation of IFN-**γ** in a positive feedback loop, but also results in the suppression of IL-4 expression, a cytokine known to promote Th2 differentiation[47]. IL-12, however, operates through a distinct STAT4-dependent pathway to enhance production of IFN-**γ**, and additionally, it triggers the upregulation of its own receptor and that of IL-18[48]. Moreover, Th1 cells have been known to produce IL-2, a proinflammatory cytokine that induces T cell proliferation, and TNF**α**, a cytokine responsible for the activation of epithelium at the site of infection[49]. Collectively, these effects result in the amplification of the Th1 response.

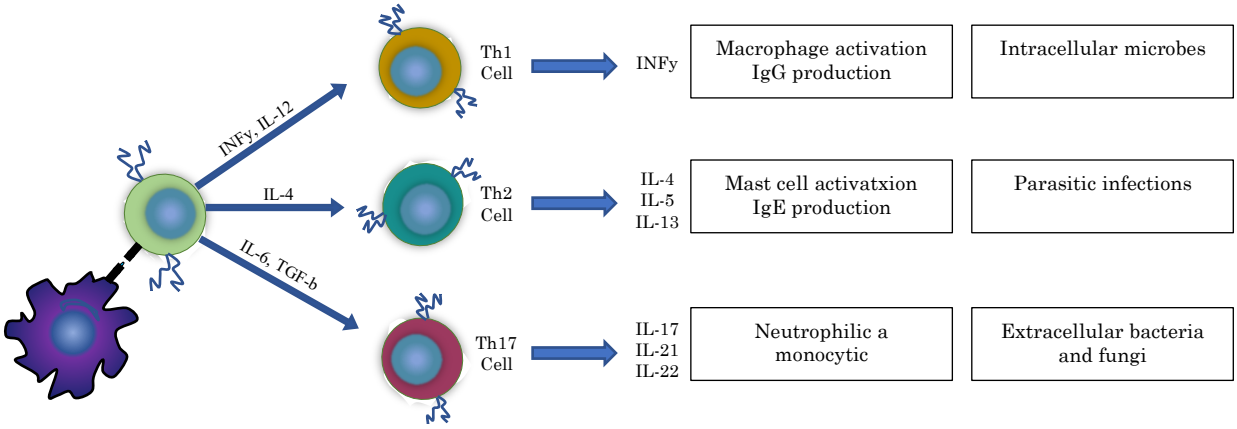


Figure 1. Differentiation of naïve CD4 T cells into Th1, Th2 and Th17 Effector Cells.

The Th2 cellular response is synonymous with targeting parasitic infection[40]. A range of Th2-associated cytokines, including IL-4, IL-5, IL-10, and IL-13, mediate the orchestration of the response[40,50]. In this setting, Th2 cells stimulate humoral immunity through the production of IL-4 by regulating the induction of B cell IgE antibody isotype switching[51]. IgE immune complexes are responsible for cross-linking high-affinity Fc receptors for IgE (Fc**ε**RI) on the surface of innate immune cells such as eosinophil, mast cells and basophils, resulting in their activation. Additionally, IL-5 stimulates the proliferation and differentiation of eosinophils, while IL-13 enhances B cell proliferation and isotype switching and inhibits the production of anti-inflammatory cytokines[52]. Once activated, basophils and mast cells secrete various

products, including histamine, serotonin and proteases, as well as several cytokines and chemokines, which result in smooth muscle constriction, vascular permeability and the recruitment of more pro-inflammatory cells[53]. Furthermore, the expression of Fc**ε**RI is further upregulated on these cells as a result of the IgE cross-linking, hence, provides a potent amplification of IgE-mediated Th2-type effector responses[54]. The generation of the Th2 response occurs when naïve $CD4^+$ T cells are exposed to IL-4 at the time of T cell priming. In low antigen conditions, responding T cells produce IL-4, however, in an activated state, innate cells such as eosinophils, mast cells and basophils also contribute to its production[50]. Recent work demonstrates that through interaction with its receptor, IL-4 activates STAT6[55]. The fundamental role of STAT6 in Th2 differentiation is further supported by a profound decline in the development of this lineage observed in Stat6-deficient mice[56]. It is now known that the activation of STAT6 leads to its nuclear translocation and subsequent induction of the transcription factor GATA3, which, like T-bet for Th1 cells, is considered the master regulator of Th2 differentiation. Thus, the mature Th2 cell's production of IL-4 acts in a positive feedback loop to promote further Th2 cell differentiation in naive T cells as they encounter antigen. Nevertheless, a Th2 response has the potential to contribute to pathologic conditions of allergy and airway hypersensitivity, when inappropriately regulated[57,58].

The Th17 type response was first described in 2005 as having a primary function to protect against extracellular bacteria and fungi[41]. Since then, many studies have further investigated their immune function and the mechanisms that regulate this function[59,60,61]. It is now understood that in the presence of IL-6 and TGF-b, but absence of IL-4 and IL-12, naïve $CD4^+$ are induced to Th17 lineage, and that their defining characteristic is the expression of the transcription factor ROR**γ**t, as well as the expression of IL-17A and IL-17F, as their classification name suggests[60]. The differentiation of Th17 cells is controlled by the transcription factor ROR**γ**t, which is considered the "master-regulator" as it directs the specific and heritable gene

expression profile of the subset[59]. Th17 cells are not only considered to be distinct from the other Th cells by their gene expression and regulation, but also in terms of their biological function. Th17 cells, by virtue of their production of IL-17, are generally thought to be pro-inflammatory and play an important role in host defence against infection, by recruiting neutrophils and macrophages to infected tissues, and although the hallmark of Th17 cells is the expression of IL-17, additional cytokines have been identified such as IL-21 and IL-22[60]. The expression of IL-21 is dependent on STAT3-mediated IL-6 signalling in Th17 cells and is thought to act in synergy with TGF**β** to promote Th17 differentiation[61]. Moreover, the IL-21 receptor, composed of IL-21R and the common **γ** chain, has been shown to be expressed by Th17 cells, and thus can act in an autocrine manner[60]. Th17-secreted IL-22, however, induces the expression of anti-microbial peptides beta-defensin-2 and beta-defensin-3 upon binding to its receptor on target cells[62]. Recently, it was demonstrated that IL-22 is able to protect hosts against bacterial infections of the lungs and gut[63]. The Th17 response has however been suggested to be complicit in autoimmune diseases[64,65].

While a considerable proportion of research has focused on T cells that recognise peptides presented in major histocompatibility complex (MHC) proteins, numerous other subtypes do not fit this paradigm. Somewhere between the rapid sensing and wide ranging innate immune system and the finely tuned repertoire of the adaptive system there exists a group of T cells that display both innate and adaptive characteristics. Collectively, they are considered "innate-like" or "unconventional" T cells, to a certain extent because they recognise lipids, small-molecule metabolites and particularly modified peptides.

Restricted repertoires appear to define these discrete lymphocyte subpopulations that exist at the frontier between the innate and adaptive immune system. This is likely due to the fact limited repertoires allow for the presence of a high number of pre-existing cells that are responsive to phylogenetically conserved antigens[66].

Alternatively, populations with a highly restricted repertoire could play an immunoregulatory or alternative physiological role[67]. Numerous types of so called "unconventional" T cells in humans have been described, including CD1 restricted T cells, which encompass the NKT cell population, mucosal associated invariant T cells, and **γδ** T cells named by their unique expression a gamma and delta chain TCR[68,69,70].

2.2 Mucosal Associated Invariant T Cells

Mucosal associated invariant T cells (MAIT cells) are a relatively recently described population of innate-like T cells[69]. They represent a remarkably abundant population in human blood and liver where they account for 1–10% and 20–45% of all T cells, respectively. After birth, human MAIT cells expand and acquire a homogeneous phenotype, however, in contrast to conventional T cells, which can display an orthodox TCR with an almost unlimited repertoire, MAIT cells express a semi-invariant TCR rearrangement with limited specificities[71]. Despite their relative abundance and unique specificity, the function of MAIT cells within the immune system has yet to be clearly defined. Nevertheless, the field is rapidly advancing, with numerous studies now suggesting the significance of these cells in disease and their potential as therapeutic targets[72,73,74,75].

2.2.1 MAIT cell Identification and Phenotype

Porcelli et al. first identified the existence of the invariant TCR**α** chain, V**α**7.2-J**α**33, while investigating TCR**α** chains of double negative T cells from peripheral blood of healthy individuals[76]. They observed the preferential pairing between the invariant TCR **α**-chain and a limited TCR **β**-chain repertoire. Following this discovery, Tilloy et al. went on to describe a homologous restricted population, with V**α**19-J**α**33 rearrangement, in mice and cattle[77]. This discovery highlighted that this unique population of T cells are phylogenetically conserved, and consequently, could serve an historic and important physiological role in mammals. Despite the identification of restricted population in 1993, it was not until 2003, that Treiner et al. noticed, in a

landmark study, the absence of V**α**19-J**α**33 transcripts in MHC class I-related (MR1) deficient mice and thus, identified the MR1, a non-polymorphic protein, as the restricting molecule of this cell population[78]. The biological significance of this interaction is accentuated by the fact the genes encoding for MR1 and the invariant TCR are highly conserved throughout mammalian evolution, with species that lack MR1 also selectively lacking a TCR**α** chain homologous to V**α**7.2-J**α**33 in humans. The populations were termed mucosal-associated invariant T cells or MAIT cells, due to their relative enrichment in the gut lamina propria of humans and mice.

Although Porcelli et al. first described the MAIT cell population in humans as $CD4^-$ $CD8^-$ T cells, by the end of 1993, A. Bendelac verified the results of S. Porcelli, with a comprehensive study showing that the human V**α**7.2-Ja33 TCR**α** chain was prevalent, not only in DN T cells, but also in the $CD8^+$ population[79]. Today, the current dogma accepts that the majority of human MAIT cells are typically $CD8^+$, and notably, they are largely $CD8\boldsymbol{\alpha}^+CD8\boldsymbol{\beta}^{-/lo}$, suggesting a predominant expression of CD8**αα** homodimers, contrasting the mainly $CD8\boldsymbol{\alpha\beta}^+$ TCRs found in the conventional T cell populations. The other 10–20% of the MAIT cell population consists of a DN population, and a CD4+ subset that represents less than 5% of all MAIT cells. The function of CD4 and CD8 in MAIT cell biology has yet to be fully understood, as there is little evidence to suggest an apparent functional or phenotypic difference between these subsets[80]. One recent study, however, has suggested that the $CD4^-CD8^-$ subset is developmentally more mature than the main $CD8^+$ pool[81]. $CD4^+CD8^-$ MAIT cells on the other hand, are often overlooked, but are thought to signify a distinct population. They have been shown to produce more IL-2 than the other MAIT cell populations, and additionally do not show the same age-related kinetics that other MAIT cells demonstrate[82], therefore, it is quite possible that they could have a distinct role within the immune system.

Phenotypically MAIT cells express both Th1- and Th17- like features and share some of the same characteristics as natural killer (NK) cells, most notably, the high

expression of the C-type lectin CD161[83]. Moreover, they also display a high expression level of CD26, a dipeptidase processing various chemokines, growth factors, and neuropeptides[84]. The expression of homing receptors, CCR6, CXCR6, CCR5, CCR2, and intermediate levels of CCR9, are present at high levels, enabling them to migrate to the liver, gut and lung[85,86]. Conversely, however these cells express very little to no CD62L and CCR7; receptors responsible for migration into non-inflamed lymph nodes[85,87]. In accordance with this, MAIT cells can be observed in the lungs of patients with infections such as M. tuberculosis, as well as in human gut samples, but are rarely detected in human lymph nodes[88]. MAIT cells also display the characteristic expression of an effector-memory phenotype $CD45RA^{lo}CD45RO^{+}$ $CD27^{+}$, yet interestingly, they are strikingly not cycling ($Ki\text{-}67^{-}$) despite their high clonal size[85,89]. Furthermore, MAIT cells display a high expression of cytokine receptors such as IL-18R**α**, IL-12R, IL-7R, and a distinctive profile of transcription factors (PLZF, ROR**γ**t, T-bet, Eomes and Helios)[90,91,92]. Finally, MAIT cells have been shown to display PLZF-dependent proapoptotic phenotype, as indicated by increased susceptibility to *in vitro*-induced apoptosis[93]. ***Figure 2*** recapitulates the phenotype of classical mucosal associated invariant T cells in the peripheral blood of healthy adult individuals.

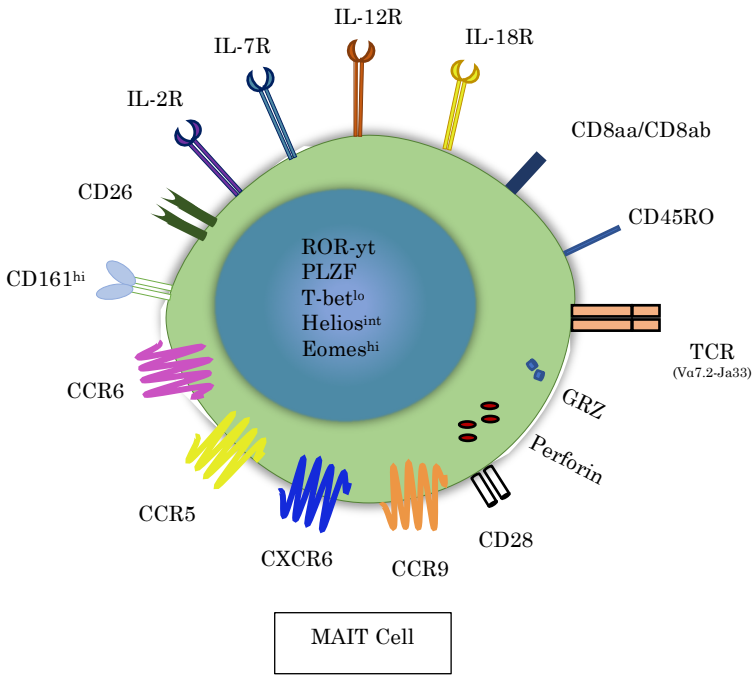


Figure 2. Phenotype of classical mucosal associated invariant T cells in the periphery of a healthy human adult. *Expression levels are abbreviated as hi (high), int (intermediate), or lo (low).*

In 2009 Martin et al. developed a monoclonal antibody (clone 3C10) that binds to the human TCR V**α**7.2 segment[94]. This breakthrough enabled the identification of V**α**7.2$^+$ cells by flow cytometry. Later in 2011, Le Bourhis et al. showed that the co-expression of the surface marker CD161 or IL-18 receptor **α** (IL-18R**α**) with V**α**7.2$^+$ cells highly enriched for the MAIT cell population[95]. The field soon adopted the use of V**α**7.2, and co-expression of high levels of CD161 within the T cell compartment as the primary method to identify MAIT cells using flow cytometry. Since then, however, the identification of MAIT cell agonists by Kjer-Nielsen et al. has led to the generation of fluorescent MR1 tetramers refolded with such compounds[96]. In healthy adult individuals, the cell population identified using MR1 tetramers greatly overlaps with those defined as CD161^{hi} V**α**7.2$^+$ [82, 97]. But It remains unclear how well these surrogate markers define these cells in other tissues or in the context of disease.

2.2.2 MAIT Cell Development

Like other T cells, MAIT cells develop in a thymus-dependent manner. However, in contrasts to conventional T cell development, in which positive selection entails the interaction between the TCR and MHC proteins expressed on thymic epithelial cells, MAIT cells are selected into the lineage by double-positive, MR1-expressing cortical thymocytes[98,101]. This occurs following the random rearrangements in TCR-encoding genes, where cells then generate a TCR capable of interacting with MR1. Several studies had initially alluded to this possibility, as they published results showing DP thymocytes that exhibit a high level of endogenous MR1 in both mice and humans[99,101]. Nevertheless, it was Seach et al. that went on to demonstrate the imperative and role of MR1-expressing DP thymocytes in MAIT cell selection, with the use of transgenic mouse models and thymic organ cultures[101]. Koay et al. went on to investigate MAIT cells in the thymus with the use of MR1 tetramer and proposed a three-stage pathway for MAIT cell development[102]. In humans this consists of stage 1 containing predominantly DP and CD4$^+$ thymocytes expressing a CD3$^+$MR1tet$^+$CD27$^-$

CD161⁻ phenotype, stage 2 comprising of mostly DP, CD4⁺ and CD8⁺ cells that display a CD3⁺MR1tet⁺CD27⁺CD161⁻ expression profile and finally stage 3, which primarily consists of CD8⁺ and DN CD3⁺MR1tet⁺CD27$^{+/low}$CD161⁺ cells. Stage 1 MAIT cells are only found in the thymus, whereas stage 2 MAIT cells have also been observed at low levels in the periphery of young children[102]. The transition from stage 2 to stage 3 involves upregulation of PLZF and IL-18R, and the acquisition of functional potential to functional maturation occurs extra-thymically. Stage 3 MAIT cells are infrequent in the thymus but predominate in cord and peripheral blood. However, there are close parallels in the development of mouse MAIT cells and those of a human MAIT cells. In the thymus, however, CD4⁺, and DP MAIT cells are represented in more substantial proportions of CD8⁺ MAIT cells[102]. A better understanding of what regulates the number and function of MAIT cells will require a clearer picture of the development and homeostasis of MAIT cells.

2.2.3 Antigen Presentation to MAIT Cells

For many years after the discovery of MAIT cells, the type of antigens they detect in association with MR1 was unknown, which was a major obstacle to understanding the biology of these cells. In 2010, two studies, Gold et al. and Le Bourhis et al., first demonstrated human MAIT cell activation by bacteria-infected cells in an MR1-dependent manner, and that they respond to a surprisingly broad range of microbial organisms, including many bacteria and yeast[95,103]. In 2012, a ground-breaking paper by Kjer-Nielsen et al. demonstrated that MR1 binds to vitamin B metabolites derived from vitamin B2 and vitamin B9[96]. These include 6-FP, folic acid, and several ribityllumazines and pyrimidine-based intermediates. Vitamin B2 had the capacity to activate MAIT cells. These results were verified with genetic studies, where the deletion of genes encoding key enzymes in the riboflavin pathway abolishes an otherwise productive MAIT cell response to certain bacteria[104]. Corbett et al. went on to define the key antigens that bind MR1 and result in MAIT cell activation as 5-OP-RU and 5-OE-RU[105]. Most of the focus since then been on 5-OP-RU, which is loaded

into MR1 tetramers to identify MAIT cells. A mounting list of ligands have been found that bind to MR1, include some pharmacological agents and their derivatives, and this flags the possibility that the MR1–MAIT cell axis may contribute to certain drug-hypersensitivity reactions[106,107,108]. Further characterisation of these antigens is needed to understand their relation to the well-defined ribityl antigens. The development of MR1-antigen tetramers now permits the specific identification of MAIT cells based on T-cell receptor specificity.

2.2.4 MAIT Cell Activation and Effector Functions

Mucosal-associated invariant T cells are considered to span both the innate and adaptive arms of the immune system. Once activated MAIT cells are triggered to promote a rapid immune response mediated by the release of cytotoxic molecules and inflammatory cytokines, such as interferon gamma (IFN**γ**), perforin, granzymes and tumour necrosis factors (TNF)[109,110]. Upon TCR-dependent recognition of microbial antigens, MAIT cells have the capacity to rapidly eliminate bacterially infected cells through the production/secretion of inflammatory cytokines such as IFN**γ**, TNF**α**, and IL-17 and cytotoxic effector molecules like perforin and granzyme B. The bacterially derived activation of MAIT cells rapidly induced Grz B and Perforin production, licensing these cells to kill their associated target cells[109]. Cytokines produced by MAIT cells not only act directly on infected target cells, but also promote the activation of other immune cell populations and thus, assists the orchestration of the adaptive immune response through approaches such as dendritic cell (DC) maturation[111,112]. MAIT cell mediation of a potent antimicrobial activity likely contributes significantly to the protection against diverse bacterial infections in humans. Importantly, van Wilgenburg, B. et al. demonstrated that human MAIT cells are also able to activate in vitro in a manner that is TCR independent but dependent on IL-18, IL-12 and IL-15 and/or interferon **α**/**β**[113]. Consequently, MAIT cells can be activated in various non-bacterial inflammatory conditions in which these cytokines

are produced. In particular during human viral infections such as dengue, influenza virus, HCV, and HIV[113]. For the same reasons, MAIT cells may participate in non-infectious pathological conditions, such as autoimmune disorders and cancer. Accordingly, in humans, MAIT cells are the most abundant T cell subset able to detect and kill bacterially infected cells.

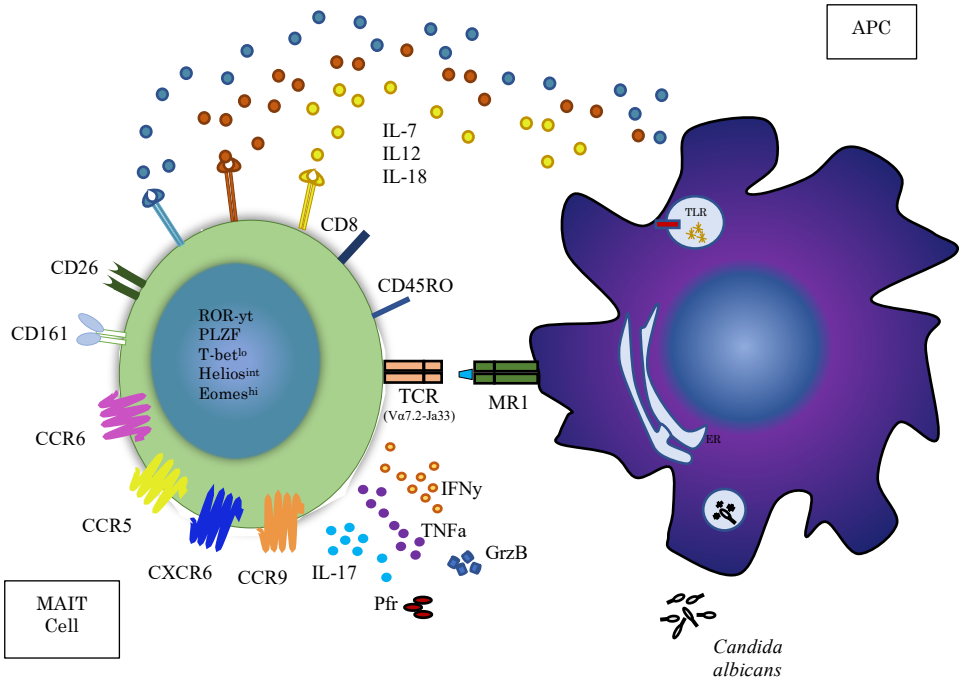


Figure 3. TCR dependent and independent MAIT cell activation. MAIT cells are able to produce IFNγ and TNFα, as well as to degranulate, release GrzA, GrzB, Gnly, and Prf, and kill target cells, in response to TCR-antigen-MR1 interactions and also from the direct effect of APC-derived innate cytokines, such as IL-12 and IL-18.

2.2.5 The Role of MAIT Cells in Infection, Autoimmunity and Inflammation

The function and significance of MAIT cells in health and disease has only started to be unravelled. It is becoming increasingly clear that mucosal associated invariant T cells are modulated not only in microbial infections but also non-microbial diseases. Thus far, MAIT cells have been implicated in various types of immune disorders, such as Multiple Sclerosis (MS), Type I diabetes (T1D), Systemic Lupus Erythematosus (SLE) and Inflammatory Bowel Diseases[114-118]. Rouxel et al., first described the alterations in MAIT cell in patients with type 1 diabetes and the regulatory role of these cells through maintaining gut integrity[116]. Moreover, Magalhaes. et al. depicted the highly

activated phenotype and strong IL-17 profile in MAIT cells of obese and/or diabetic patients[119]. Furthermore, the relevance of MAIT cells in the context of therapeutic applications were highlighted by Croxford et al., when they reported MAIT cell inhibition of autoimmune disease in the context of experimental autoimmune encephalomyelitis[120]. Likewise, several studies have pointed to a potential involvement of MAIT cells in tumour immunity[121]. Although their contribution to each of these pathologies are currently still undefined. Nevertheless, it is clear that circulating MAIT cells are dramatically reduced in most diseases and hence, elucidating the regulatory mechanisms of MAIT cell activation could have clinical implications.

There is presently a great deal of interest in the identification of other MR1-binding antigens, particularly mammalian derived antigens that may link MAIT cells to self-reactivity and tumour immunity. Intrathymic MAIT cell selection is MR1 dependent, evident also in germ-free mice, thus the selecting antigen is likely not of microbial origin[121]. Moreover, selected cells, including some human B cells or tumour cell lines, express cell-surface MR1 in the apparent absence of microbial antigens[122]. This evidence together with the suggestions that non–riboflavin-based antigens can bind to MR1 and activate some MR1-restricted T cells[123], implies that the MR1 ligands are even more diverse than is currently appreciated.

2.3 The Transcription Factor Helios

2.3.1 IKAROS Family of Transcription Factors

The transcription factor Ikaros, encoded by the Ikzf1 gene, was first described in 1992 by Georgopoulos et al., by virtue of the protein ability to bind and activate the enhancer encoding for the T cell differentiation molecule, CD3**δ**[124]. Following this discovery, four other proteins, Helios, Aiolos, Eos and Pegasus, that shared a high degree of homology to Ikaros, were identified. These DNA-binding proteins now comprise the Ikaros family of transcription factors and are characterised by the

presence of two sets of highly conserved Krüppel-type zinc finger motifs[125]. One set, located at the amino (N)-terminus, mediates binding to specific DNA sequences, while the other, found at the carboxy (C)-terminus, enables the protein to dimerise with isoforms and splice variants of itself or alternatively with other family members. This distinct structure grants the diverse functional capabilities that have been observed by the Ikaros family member proteins, for instance their ability to both positively and negatively regulate gene expression through the direct interaction with DNA, as well as by forming transcriptional complexes with other proteins[126]. It is believed that the family of proteins are able to regulate gene transcription through chromatin remodelling, where they interact in a major transcriptional corepressor complex called nucleosome remodelling and histone deacetylase (NuRD) complex[127]. Consequently, this results in nucleosome-disruption and histone deacetylase activity that condenses chromatin and most commonly serves to repress the transcription of downstream genes. However, the mechanisms of action of these transcription factors have yet to be fully defined.

Existing knowledge of the Ikaros family indicates that these transcription factors are predominantly implicated in lymphocyte differentiation, but also play a crucial role in a wide range of processes that maintain a functional immune system, such as apoptosis, cell cycle arrest, proliferation, and differentiation[128]. Deletion of the Ikaros activation and dimerisation domains in mice resulted in a lack foetal T cells, B cells, and NK cells, and defective **γδ** T cells and dendritic cells in adult T cells, due in part to the inability of hematopoietic stem cells to differentiate into lymphoid progenitors in mice null for Ikaros[129]. Moreover, several heterozygous mutations associated with the DNA-binding domain of Ikaros have been shown to cause a common variable immunodeficiency-like syndrome with a striking decrease in B cells and NK cells, in addition to T cells, **γδ** T cells, and dendritic cells[130]. Additionally, Asanuma et al. 2013 and Georgopoulos 2017 demonstrated the existence of an association between several haematological malignancies and the atypical expression of both Ikaros and

Helios; Ikaros in B cell acute lymphoblastic leukaemia (ALL), and Helios in adult T cell leukaemia and acute myeloid leukaemia (AML)[131,132]. Thus, emphasising the crucial role this family of transcription factors play in the function and regulation of a normal immune response.

2.3.2 Expression of Helios in T Cells

Helios, encoded by the *IKZF2* gene, is expressed in several cell types, but is typically limited to the T cell lineage. Originally, Helios was identified as a protein that was highly expressed in both murine and human T regulatory cells (Tregs), and consequently, much of the research had been focused on its role in the peripheral immune tolerance[133,134]. It was initially postulated that the Helios protein differentiated natural Tregs, derived in the thymus, from their peripheral counterparts. However, since then Helios expression was shown not to be restricted to the natural Treg population, as low-level expression is also manifest in effector T cells under certain conditions[135]. These observations compellingly suggest that conventional T cells expressing Helios could sustain Helios expression when differentiating into induced Tregs in the periphery.

Currently, there is an accumulating amount of evidence to support the view that Helios plays a major role in stabilisation of the non-inflammatory phenotype of Tregs and thwarts the production of IL-2 in Tregs by epigenetic silencing[136]. In support of this evidence Nakagawa et al. 2016 and Himmel et al. 2013 showed that in selective knock-out models of human memory Tregs, the Helios^{-} Tregs produce more proinflammatory cytokines than their Helios-expressing equivalents[137,138]. Conversely, Cai et al. 2009 demonstrated that young Helios knock-out mice show no clear immunological phenotype, however, a large fraction of these homozygous pups perished for unknown reasons before weaning[139]. Later in 2016, Sebastian et al. showed that the suppressive capacity of Tregs in Helios$^{-/-}$ mice is not severely affected, and that older Helios$^{-/-}$ mice develop only a mild autoimmune phenotype,

characterised by autoantibodies and dysregulated germinal centre reactions[140]. Consequently, the role of Helios in Tregs still remains elusive. Several studies have aimed to ascertain the factors that control the expression of Helios, and thus, have identified that in addition to the constitutively expressed Helios in Tregs, the expression could also be induced upon TCR-mediated stimulation[141]. Interestingly, this has been observed not only in Tregs but also in effector T cells. It is thus evident that Helios has a significant role in regulating effector T cell activity during immune responses. Other factors controlling the expression still remain unidentified, but the involvement of NFkappaB transcription factor has been suggested[142].

So far, there has been no published evidence of a germline Helios variant that results in an immunodeficiency phenotype in humans. However, unpublished research conducted in our laboratory have described patients from a single family that display a heterozygous Helios loss-of-function mutation with an immunological deficiency-like phenotype, which echoes that of the Helios knock-out mice phenotype; lymphadenopathy with dysregulated germinal centres and aberrations in antibody production and augmented IL-2 production from both effector and regulatory T cells. These results highlight the importance of Helios' protein binding domains in mediating its functions in both effector and regulatory T cells.

2.3.3 Helios and MAIT Cells

Mucosal associated-invariant T cells are another population of T cells that express relatively high levels of Helios, however, very little is known about its function in these cells. Research conducted by Simo Miettinen in our laboratory, demonstrates that the expression of Helios in MAIT cells is in fact upregulated upon microbial stimulation, indicating that Helios is implicated in the regulation of MAIT cell activation and proliferation[143]. No difference in the levels of Helios expression between CD8, DN and CD4 peripheral MAIT cell subsets can be observed, therefore despite the

possibility that these subsets of peripheral MAIT cells could have discrete functional roles, Helios expression is common to all of them[144].

In 2017, Gibbs et al. published a study on MAIT cells from the female genital tract and their findings showed that some women had elevated Helios transcription factor in MAIT cells in the endometrial region, but not in the cervical region or blood. These same cells were stimulated with E. coli and observed for activation markers. These Helios^{++} cells exhibited an increase in IL-17 secretion that was more pronounced than in similarly stimulated blood cells[145]. However, whether Helios affects IL-17 elevation remains unclear in the study, for technical reasons. Normally, IL-17 is associated with fungal infection immune responses. Moreover, the Helios haploinsufficiency patients that were studied in our laboratory showed a reduced number of MAIT cells in their circulation, which was not occurring as a result of an increased accumulation to the gut mucosal sites. This highlights the importance of the Helios within the MAIT cell population. Thus, the role of Helios in MAIT cells is currently unclear but warrants further research in this field.

3 HYPOTHESES

We hypothesise that the transcription factor Helios, plays a functional role in regulating the activation of mucosal associated invariant T cells, and that the lack of this protein would cause a phenotypic change in the levels of effector molecules expressed by MAIT cells.

4 AIMS and OBJECTIVES

The aim of this master's thesis is to characterise the functional role of Helios in peripheral mucosal associated invariant T cells by using siRNA to silence Helios expression, and flow cytometry to analyse any potential alterations in MAIT activation markers. The objectives of this project are as follows:

1. To validate the upregulation of Helios upon CD3/CD28 stimulation in MAIT cells.

2. To determine the time point where expression of Helios is at its highest-level following stimulation, and thus, the optimal time point at which to analyse the potential changes in protein expression of MAIT cells with reduced Helios expression.

3. To attenuate the expression of Helios in MAIT cells by at least 50% using targeted siRNA.

4. To evaluate differences in the expression of MAIT cells activation markers that occur as a result of the knockdown in Helios.

5 MATERIALS and METHODS

5.1 Sample Collection and Cell Isolation

Venous blood samples were drawn into 10 mL Heparin Vacutainers (Beckton Dickinson (BD), San Jose, CA) from healthy adult volunteers and rotated from end to end several times. Alternatively, buffy coats were obtained from the Finnish Red Cross Blood Service (Helsinki, Finland). Donor 1: Male, 57. Donor 2: Male, 60. Donor 3: Male, 36.

5.1.1 PBMC Isolation

Peripheral blood mononuclear cells (PBMCs), the fraction containing the lymphocyte population, were isolated using a Ficoll-Plaque (GE Lifesciences, Uppsala, Sweden) gradient. Several 50mL falcon tubes containing 15 mL of Ficoll were left to warm to room temperature (RT). Meanwhile, the blood collected in each Vacutainer was transferred to a clean 50mL falcon and diluted in a 1:3 ratio with room temperature (RT) sterile phosphate-buffered saline (PBS). The 30mL of the diluted blood was then very carefully overlaid onto each 15mL Ficoll, creating a distinct separation between the two layers. The buffy coat, however, was diluted in the bag at a 1:6 ratio with sterile PBS at RT, before being transferred in 30mL portions to clean 50mL falcon tubes, and then overlaid onto the Ficoll. The falcon tubes were gently placed into a swinging bucket rotator centrifuge, and centrifuged for 30 minutes with the break off and accelerator set to the minimum, at 1500 rpm, RT. Once centrifuged, the interphase fraction could be vigilantly removed, using a pastor pipette, into a fresh 50mL falcon tube and washed twice in ice cold PBS for 10 minutes at 1200 rpm, +4°C. Following the final wash, the cell pellet was resuspended in an appropriate volume of sterile PBS for automated cell counting (TC20, Bio-Rad).

5.1.2 Enrichment of CD3+ or Vα7.2+ Cells

Unfractioned PBMCs or enriched CD3+ cells were used to optimise the conditions for transfection. Lonza has optimised the transfection settings used for unstimulated T cells, and thus, when transfecting PBMCs on these settings, it caused cell death of the other mononuclear cell populations, and clumping was evident in the culture. Therefore, in future optimisation experiments, the PBMCs were first enriched for $CD3^+$ cells prior to transfection. For the experiments that evaluated the knockdown of Helios in MAIT cells, we enriched the PBMCs from the buffy coats obtained from the Finnish Red Cross Blood Service, for the TCR V**α**7.2 to increase the sample throughput. Purification of $CD3^+$ T cells or V**α**7.2^+ T cells from freshly isolated PBMCs was performed using the Pan T Cell or Anti-PE Microbead isolation kit (Miltenyi Biotec) respectively, with LS Columns in a MidiMACS Separator (Miltenyi Biotec). For V**α**7.2 enrichment, up to 10^7 PBMCs per 0.1 mL of FACS staining buffer (PBS, pH 7.4, 10% heat-inactivated FBS, and 2 mM EDTA) were incubated for 30 minutes at +4°C with a V**α**7.2 primary antibody conjugated to PE (Biolegend). The cells were washed in 1-2mL of MACS buffer (PBS, pH 7.2, 0.5% heat-inactivated FBS and 2 mM EDTA) per 10^7 cells for 10 minutes, at 400 g, in +4°C. Once the supernatant had been aspirated, the PBMCs were then resuspended in 80 µl MACS buffer followed by 20 µl of the Anti-PE MicroBeads per 10^7 total cells and incubated for 15 minutes in the dark at +4°C. The cells were washed again and finally resuspended in 500 µL of MACS buffer for up to 10^8 cells. When higher cell numbers were used, the volumes were scaled up accordingly. For Pan T cell isolation, PBMCs were resuspended in 40 µl, per 10^7 cells, in MACS buffer and 10 µl of Pan T Cell Antibody Cocktail is added, so that the total volume per 10^7 cells is 50 µl and incubated for 5 minutes in the dark at +4°C. 30 µl of MACS buffer was then added to the cell-antibody mix, followed by 20µl of the Pan T Cell MicroBead Cocktail per 10^7 cells, and incubated for 10 minutes in the dark at +4°C. The cells are washed and resuspended in the same manner as the V**α**7.2 enrichment protocol. Meanwhile, the LS columns (Miltenyi) were prepared

by rinsing them with 3 mL of MACS buffer. The cell suspension could then be placed into the column while positioned in the MidiMACS Separator (Miltenyi), and the flow-through for the pan T cell enrichment (containing the $CD3^+$ cells) was collected. However, as the V**α**7.2 enrichment is positive selection, the flow-through could be discarded. In this case, the column was removed from the magnetic separator, and 5mL of MACS buffer was applied to the column, before immediately flushing the magnetically labelled cells into a collection tube. The enriched cells were then counted using the automated cell counter (TC20, Bio-Rad), and 100,000 cells were aliquoted for FACS staining.

5.2 Nucleofection

Nucleofection is an electroporation-based transfection method that enables the transfer of nucleic acids, such as DNA and RNA, into cells by applying a specific voltage and reagents. This technique was chosen to transfect the siRNA, as it has been specially developed to obtain an optimal result in traditionally 'hard-to-transfect' cell types, such as primary cells. AllStars Hs Cell Death Control, AllStars Negative Control and Helios FlexiTube GeneSolution siRNAs (Qiagen) were rehydrated according to the instruction of the manufacturer. For nucleofection, freshly isolated CD3+ or V**α**7.2+ cells were aliquoted to approx. $2.5x10^6$ cells per Eppendorf and washed in PBS following their enrichment (centrifuged for 6 minutes, at +4°C and 400g) before being resuspended in 100µl of Nucleofector Solution Provided in the P3 Primary Cell Transfection Kit (Amaxa, Cologne, Germany). The nucleofector reagents have been adapted specially to stabilise this particular cell-type during nucleofection and creates the conditions needed for direct transfer of genetic material into the nucleus during the electroporation. Subsequently, 2 µg of pmaxGFP vector (also provided in the P3 Kit by Amaxa) or the appropriate concentration of (positive, negative or pooled Helios FlexiTube GeneSolution) siRNA were added. In the case that the transfection efficiency could not be evaluated with

the AllStars Hs Cell Death Control, siGLO 6-FAM (Horizon) was used. The cell solution was then transferred into an electroporation cuvette and placed in the Amaxa™ 4D-Nucleofector device. Mock control experiments were performed by processing the cells in the same way, but without adding the GFP vector or siRNA to the cells. Two programmes, FI-115 (high efficiency), and E0-115 (high functionality) were compared by evaluating their transfection efficiency with GFP and cell survival. These programs differ in the strength of the electric field and the length of the electrical pulses (additional details are the proprietary information of Amaxa Biosystems, Cologne, Germany). Immediately after nucleofection, 500µl of pre-warmed IMDM media supplemented with 10% heat-inactivated FBS was added to the cuvettes. Using the small pastor pipettes provided in the kit were used to transfer the cells to a 24 well plate containing 1.5 mL of pre-warmed IMDM media supplemented with 10% FBS so that they were seeded at approx. $1.25x10^6$ per mL.

5.3 Cell Culture and Stimulation

The cells were then placed in an incubator at +37°C, 5% CO_2, for 15 hours (overnight). The media was replaced with fresh IMDM media supplemented with 10% heat-inactivated FBS and in addition 1X Penicillin-Streptomycin (Sigma-Aldrich). Immediately after, the cells were then stimulated using Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation (ThermoFisher), that had been washed in PBS, pH 7.4 with 0.1% bovine serum albumin (BSA) and 2 mM EDTA, at a 1:2 ratio of beads to cells. For cytokine stainings, 1µl of GolgiPlug (BD Biosciences) per mL of culture was then added for the last 4 hours of stimulation to enhance the detection of the cytokines, after which the cells were collected for staining.

5.4 Flow Cytometry

Techniques like flow cytometry analysis allow the quantification of several parameters from individual cells, and thus, permit the characterisation of cell size, structure,

specific phenotype, and function of millions of cells in a multi-dimensional way. Moreover, Flow cytometry is a convenient method for analysis on a single-cell level. However, a substantial limitation of this technique is the relatively small number of parameters that can be assessed simultaneously, and consequently, only a restricted number of markers that are of interest can be evaluated of the 3-laser flow cytometer. In the case of our experimental setup, flow cytometry allows us to validate the knockdown (KD) of Helios at a single-cell level, whilst simultaneously discriminating between cell populations and displaying the activated phenotype in Helios$l^{ow/-}$ MAIT cells.

5.4.1 Panel Design and Set Up

The antibodies used for this project were direct fluorochrome conjugates: V**α**7.2–PE, V**α**7.2-PE-CF594, IL-17-PE-CY7, TNF**α**-BV711, and CD161-BV605 from Biolegend, Helios-eFluor and Granzyme B-APC from Invitrogen, CD8-FITC from ImmunoTools, and CD3–BV786, CD4-APC-CY7, Ki-67-FITC, CD25-PE-CF594, and IFN**γ**-AF700 from BD Bioscience. LIVE/DEAD Fixable Aqua Dead Cell Stain or LIVE/DEAD™ Fixable Green Dead Cell Stain were used to identify and exclude dead cells within the samples. An MR1 tetramer directly conjugated to APC (kindly donated by the NIH Tetramer Core Facility, Emory University Vaccine Center, 954 Gatewood Road, Atlanta, GA, 30329) was used to compare the use of surrogate markers in healthy donors for identifying MAIT cells. The MR1 tetramer technology was developed jointly by Dr. James McCluskey, Dr. Jamie Rossjohn, and Dr. David Fairlie, and the material was produced by the NIH Tetramer Core Facility as permitted to be distributed by the University of Melbourne.

When using multiple fluorochromes in an experiment, several factors may impact the quality of the data. The most crucial is discerning which fluorochrome should be matched with which antibody. This is because within the collection of fluorochromes that can be used with our specific flow cytometer there is a wide range of intrinsic

brightness among them, and some antigens are highly expressed while others are expressed at lower levels. Additionally, fluorescent emissions from one fluorochrome often have spillover into another detector that is designed to measure the signal from a different fluorochrome (***Figure 4***), and thus interferes with that signal. The higher the spill over, the lower the resolution, and therefore, the panel design should reflect the context of the co-expression of antigens on the cell type of interest, to minimise the potential for spectral overlap. In order to do this, wherever possible, the brightest fluorochromes were matched with the dimmest antigens, and co-expressed markers were spread across different lasers.

Single stain compensation controls are an essential part of an accurate experimental setup for multicolour assays. With the use of proper compensations, complex data sets from multicolour assays can be visualised and correctly analysed with a well-designed panel. Single-stained controls for each fluorochrome were prepared using one drop of BD Bioscience CompBeads (BD, San Jose, CA), and the addition of the single antibody-fluorochrome conjugate to each tube and incubated for 30 minutes in the dark at +4°C.

Photomultiplier tubes (PMTs) are the most commonly used detectors for flow cytometry. They reside in each channel of the instrument and convert energy of a photon into an electronic signal, which can then be amplified by turning up the voltage going through the PMT. In setting up the PMT voltages for a new panel, several factors were considered. Firstly, minimising the electronic noise in the data to ensure that it does not interfere with the measurements of dim or negative events. To do this, the minimum PMT voltages settings were determined by calculating approximately 2.5X the robust standard deviation of the electronic noise with the cell type of interest. Secondly, in order to ensure that the brightest population of the stained cells remain within the linear range of the detectors a sample of fully stained cells should be run so the brightness of each fluorochrome can be assessed. And

lastly, a reasonable resolution of the dim populations at the selected voltages should be confirmed in the fully stained cells. Once the PMTs had been determined, the single-stained BD CompBeads were checked to confirm that each bead is brightest in its primary detector and was then recorded. Finally, the application settings were saved to ensure these settings would remain when running the samples at future time points.

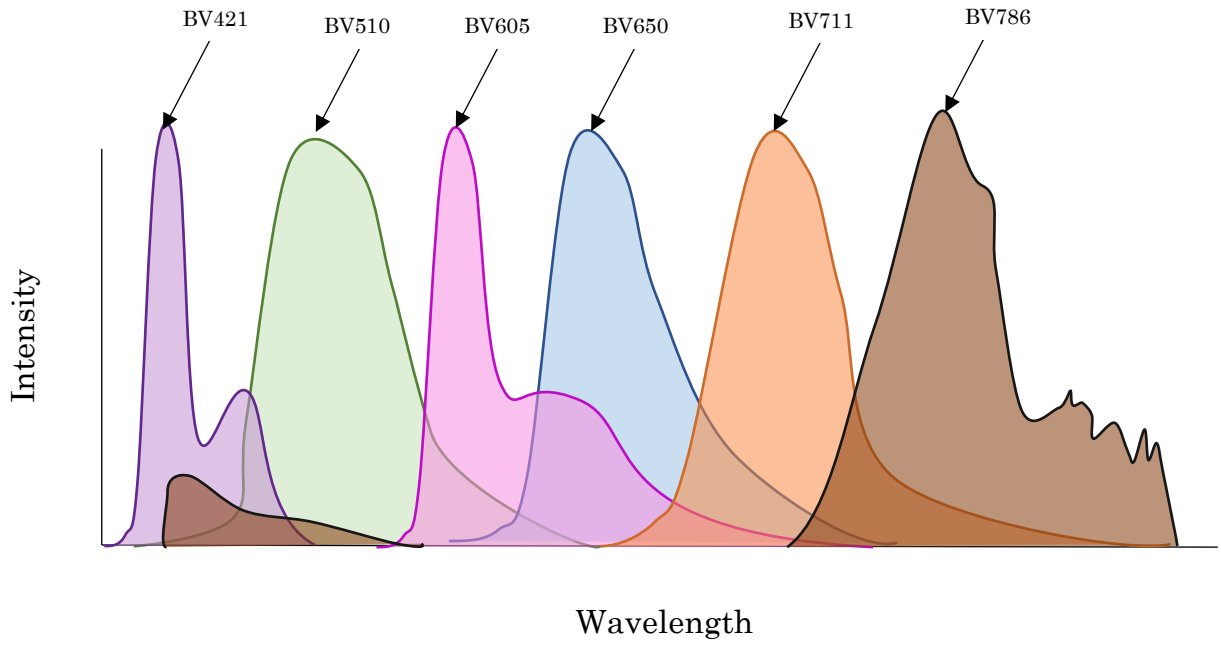


Figure 4. Spectral Overlap of the Violet Laser. Emission spectra of BV421 and its tandems, BV510, BV605, BV650, BV711, BV750, and BV785, excited by the 405nm laser.

5.4.2 Surface Stainings

For staining of surface antigens, the T cells were harvested, and the CD3/CD28 Dynabeads were removed with a magnet prior to the staining and washed in 1mL of FACS staining buffer. The antibodies targeted to surface antigens were prepared in a master mix in a 50µl volume of Brilliant Stain Buffer (BD Bioscience) per sample (***Tables 1-5***). Live/Dead Fixable Dead Cell Stain was then added to the master mix just prior to adding to the cells. The cell pellets were then resuspended in 50µl of the antibody master mix per sample and incubated for 30 minutes at +4°C, and in the case of no further intracellular staining, the cells were washed once more before resuspending in 250µl of FACS staining buffer. When applicable, the cells were

incubated with MR-1 tetramer (1:2500) in +37°C for 45 minutes and washed before continuing to other surface markers.

5.4.3 Intracellular Stainings

For staining of transcription factors, Ki67 and cytokines, the cells were fixed and then permeabilised after surface staining with FoxP3 Transcription Factor Staining Buffer Set (eBioscience). Before fixation, the cells are washed in 1mL of FACS staining buffer to remove any of the excess surface antibodies. Meanwhile, 1-part Permeabilisation Concentrate was diluted with 3-parts diluent. The cell pellets were then resuspended in 500µl of the fixation solution per sample and incubated in +4°C for 45 minutes. To permeabilise the cells, they were washed twice in 1X permeabilisation buffer. In the meantime, the intracellular antibody master mix was prepared in a 50µl volume of 1X permeabilisation buffer per sample (***Table 1 and 5***). The cell pellets were then resuspended in 50µl of the antibody master mix per sample and incubated 30 minutes at +4°C. Finally, the cells were washed in 1mL of 1X permeabilisation buffer before, resuspending in 250µl of FACS staining buffer, ready for running.

5.4.4 Table of Flow Panels

Several panels were set up for the different experiments conducted for this project. The following tables represent the panels for each series of experiments depicted in the results section. In the final experiments where the buffy coats were used to enrich for MAIT cells, three separate panels were applied to analyse several components of the experiment. First, cells that had just been enriched for V**α**7.2 were stained with the panel in ***Table 3*** to ensure that the enrichment was successful. Second, a separate panel, to check the transfection efficiency, was utilised for GFP transfected MAIT cells (***Table 4***). This was due to the fact that the signal from GFP was no longer detectable with the fixation and permeabilisation and thus, could not be incorporated into the final panel (***Table 5***), which was used to assess the knockdown of Helios and

the phenotype of the Helios$^{low/-}$ MAIT cells. The optimal concentrations for antibodies were titrated with live PBMCs.

Table 1. *Helios Upregulation Post Stimulation Panel*

Fluorochrome	**Target Antigen**	**Dilution**
FITC	DCM	1:200
PE-CF594	Vα7.2	1:20
BV421	Helios (Intracellular)	1:20
BV605	CD161	1:20
BV786	CD3	1:50

Table 2. *MR1 Panel*

Fluorochrome	**Target Antigen**	**Dilution**
FITC	DCM	1:200
PE-CF594	Vα7.2	1:20
APC	MR1 Tetramer	1:2500
BV605	CD161	1:20
BV786	CD3	1:50

Table 3. *Vα7.2 Enrichment Panel*

Fluorochrome	**Target Antigen**	**Dilution**
FITC	CD8	1:50
PE	Vα7.2	1:20
APC-CY7	CD4	1:100
BV510	DCM	1:200
BV605	CD161	1:20
BV786	CD3	1:50

Table 4. *GFP Panel*

Fluorochrome	**Target Antigen**	**Dilution**
FITC	GFP	N/A
PE	Vα7.2	1:20
BV510	DCM	1:200
BV605	CD161	1:20
BV786	CD3	1:50

Table 5. *MAIT Phenotype Panel*

Fluorochrome	Surface Target Antigen	Intracellular Target Antigen	Dilution
FITC		KI-67	1:25
PE	Vα7.2		1:20
PE-CF594	CD25		1:50
PE-CY7		IL-17A	1:20
APC		GRZ B	1:50
AF700		IFNΓ	1:50
APC-CY7	CD4		1:100
e-Fluor		HELIOS	1:20
BV510	DCM		1:200
BV605	CD161		1:20
BV711		TNFα	1:50
BV786	CD3		1:50

5.4.5 Fluorescence Minus One Control (FMO)

The fluorescence minus one control or FMO was performed as a control in the panel that was used to evaluate the knockdown efficiency of Helios in MAIT cells and any change in their activated phenotype. This control ensures that gates were placed correctly, in the context of data spread due to the multiple fluorochromes. PBMC isolation and V**α**7.2 enrichment was performed from a buffy coat as described above. $4x10^6$ enriched V**α**7.2 T cells per transfection were transferred to a cuvette and mock-transfected in the same FI-115 setting, in which the samples were transfected with siRNA or GFP vectors. 500µl of supplemented IMDM media was added, as before, and the cells were seeded at approx. $1.5x10^6$ per mL in a 24-well plate and incubated overnight, before the media was replaced and the cells were stimulated in the same manner and timeframe as the experimental samples. However, these cells were aliquoted to 11 Eppendorf tubes approx. $1x10^6$ cells per tube and stained with a cocktail of antibodies minus one per sample, as represented in the table below (***Table 6***). This allows for the assessment of problems that can potentially arise from an unforeseen spillover or unexpected interaction between dyes. The PE FMO could not be performed due to the prestaining of V**α**7.2 PE that was used to enrich for MAIT cells

Table 6. *– FMO*

Fluorochrome	FITC	PE-CF594	PE-CY7	APC	AF700	APC-CY7	BV421	BV510	BV605	BV711	BV786
FITC	-	+	+	+	+	+	+	+	+	+	+
PE	+	+	+	+	+	+	+	+	+	+	+
PE-CF594	+	-	+	+	+	+	+	+	+	+	+
PE-CY7	+	+	-	+	+	+	+	+	+	+	+
APC	+	+	+	-	+	+	+	+	+	+	+
AF700	+	+	+	+	-	+	+	+	+	+	+
APC-CY7	+	+	+	+	+	-	+	+	+	+	+
BV421	+	+	+	+	+	+	-	+	+	+	+
BV510	+	+	+	+	+	+	+	-	+	+	+
BV605	+	+	+	+	+	+	+	+	-	+	+
BV711	+	+	+	+	+	+	+	+	+	-	+

5.4.6 Analysis

The samples were run using LSR Fortessa (BD Biosciences) and analysed with FlowJo (BD Biosciences, LLC). GraphPad Prism software (GraphPad Software, San Diego, USA) was used for statistical analysis. The gating strategy to identify MAIT cells is represented in the figure below (***Figure 5***).

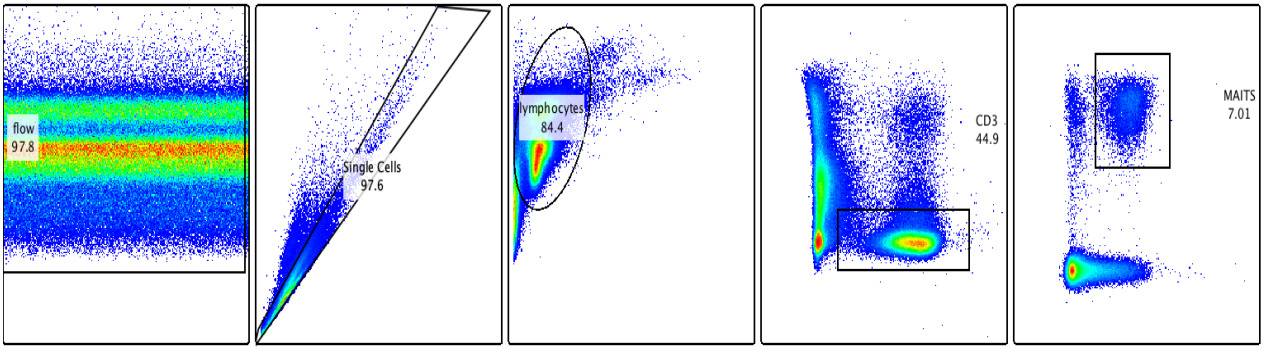


Figure 5. Gating Strategy for the Identification of Mucosal-associated Invariant T Cells in Flow Cytometry. SSC: Side scatter, FSC: Forward scatter, DCM: Dead cell marker.

5.5 Experimental Pipeline

After optimisation, an experimental pipeline was developed for evaluating differences in the protein expression of MAIT cells following the knockdown of Helios expression (***Figure 6***). Utilising the methods previously described, in the series depicted below, the ultimate aim in this project was to evaluate any differences in the protein expression of MAIT cell activation markers that are a result of the knockdown in Helios, could be achieved.

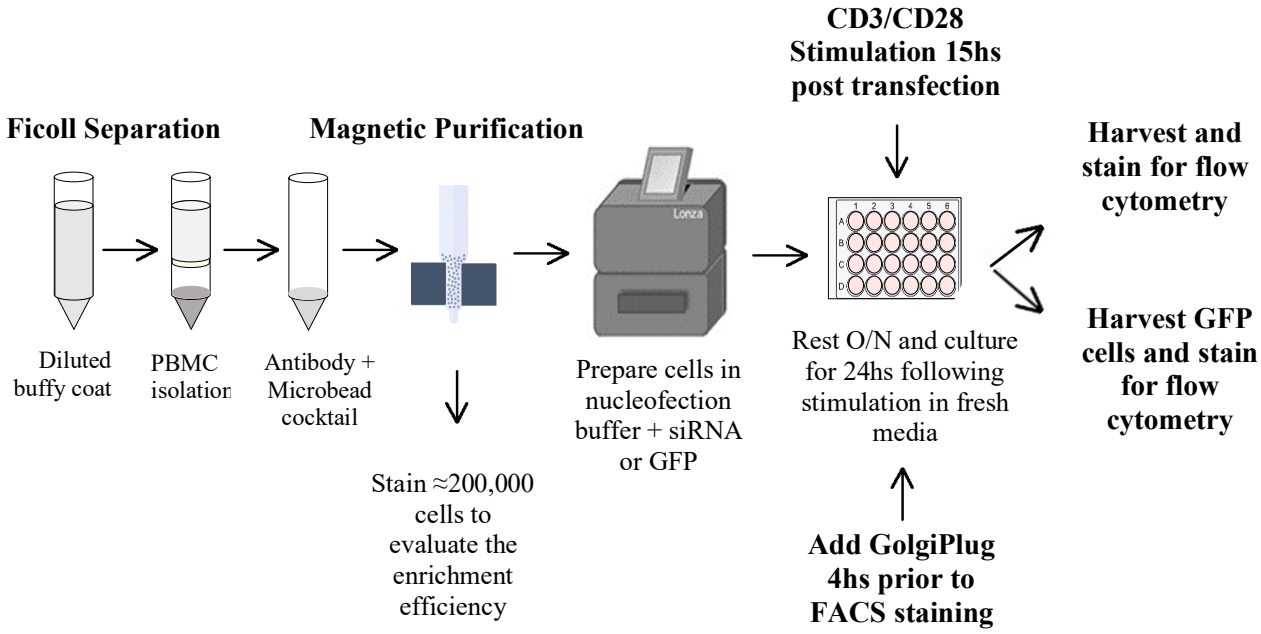


Figure 6. Experimental pipeline used to evaluate the changes in activation marker expression levels of MAIT cells, which occur as result of the knockdown in Helios.

5.6 Ethical Considerations

Sample collections for the samples from healthy donors follow the Declaration of Helsinki, and the relevant EU legislation. The protocols have been reviewed and approved by local ethical committees (Ethical Licence: HUS/747/2019).

6 RESULTS

6.1 Vα7.2 and CD161 as Surrogate Markers for MR1

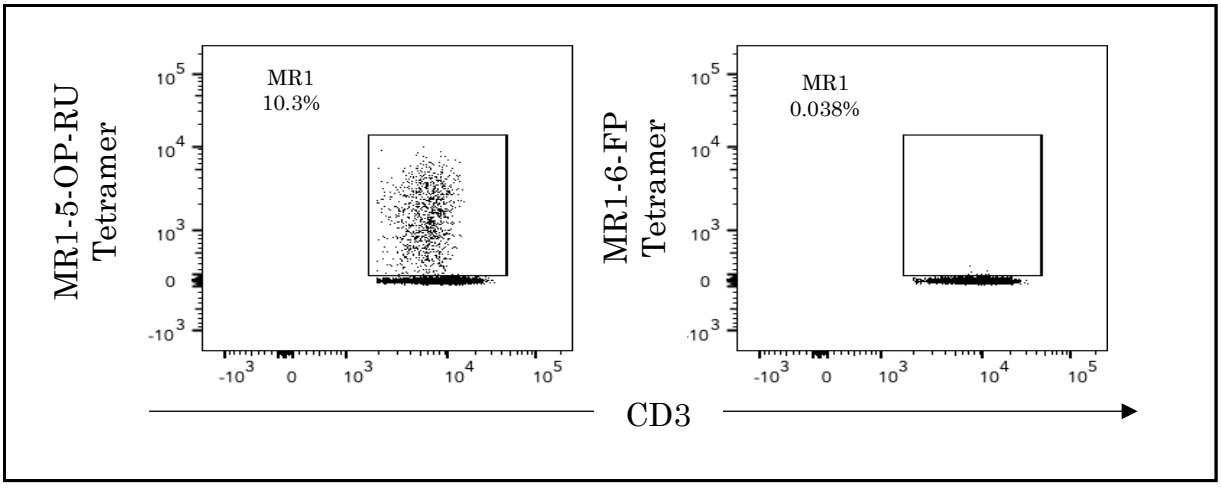


Figure 7. Identification of MAIT cells from a healthy donor using a MR1 loaded tetramer
Human MR1 tetramer pre-loaded with 5-OP-RU (left) compared to human MR1 tetramer pre-loaded with 6-FP (negative control; right), APC labelled, and stained on human PBMCs. A population of MAIT cells is clearly visible following staining with MR1-5-OP-RU tetramer (black gate). MAIT were identified by first gating for Flow/Singlets/Lymphocytes/Live/$CD3^+$ cells before plotting CD3 vs MR1 tetramer.

An MR1 tetramer loaded with 5-(2-oxopropylideneamine)-6-D-ribitylaminouracil (5-OP-RU), but not 6-formylpterin (6-FP), specifically binds MAIT cells. To identify MAIT cells in the peripheral blood of healthy individuals, we performed flow cytometric analysis of PBMCs stained with MR1 tetramer loaded with 5-OP-RU, and as a negative control, MR1-6-FP tetramer (***Figure 7***). A clear MAIT cell population is visible in this healthy donor (10.3% of total T cells), as specified by the MR1-5-OP-RU loaded tetramer staining.

Prior to the discovery and production of the MR1 loaded tetramers, the unique cell surface phenotype of MAIT cells, consisting of high expression levels of the C-type lectin CD161, the IL-18R**α** chain, as well as the ectopeptidase CD26, were often used to identify these cells in combination with a monoclonal antibody (mAb) directed against the V**α**7.2 TCR segment. One recent study examined how well these markers define the MAIT cells population in healthy individuals, of specific interest to them, was whether all MAIT cells express these markers and whether all T cells identified by

these markers are MAIT cells[97]. Their findings suggested that identification of MAIT cells using V**a**7.2 and CD161, IL-18R**a** or CD26 mAbs are largely consistent with results derived from MR1 tetramer stainings in healthy donors. To confirm these findings in our hands, we investigated how accurately CD161 and V**a**7.2 markers identify MR1-5-OP-RU tetramer$^+$ MAIT cells in three healthy individuals (***Figure 8***).

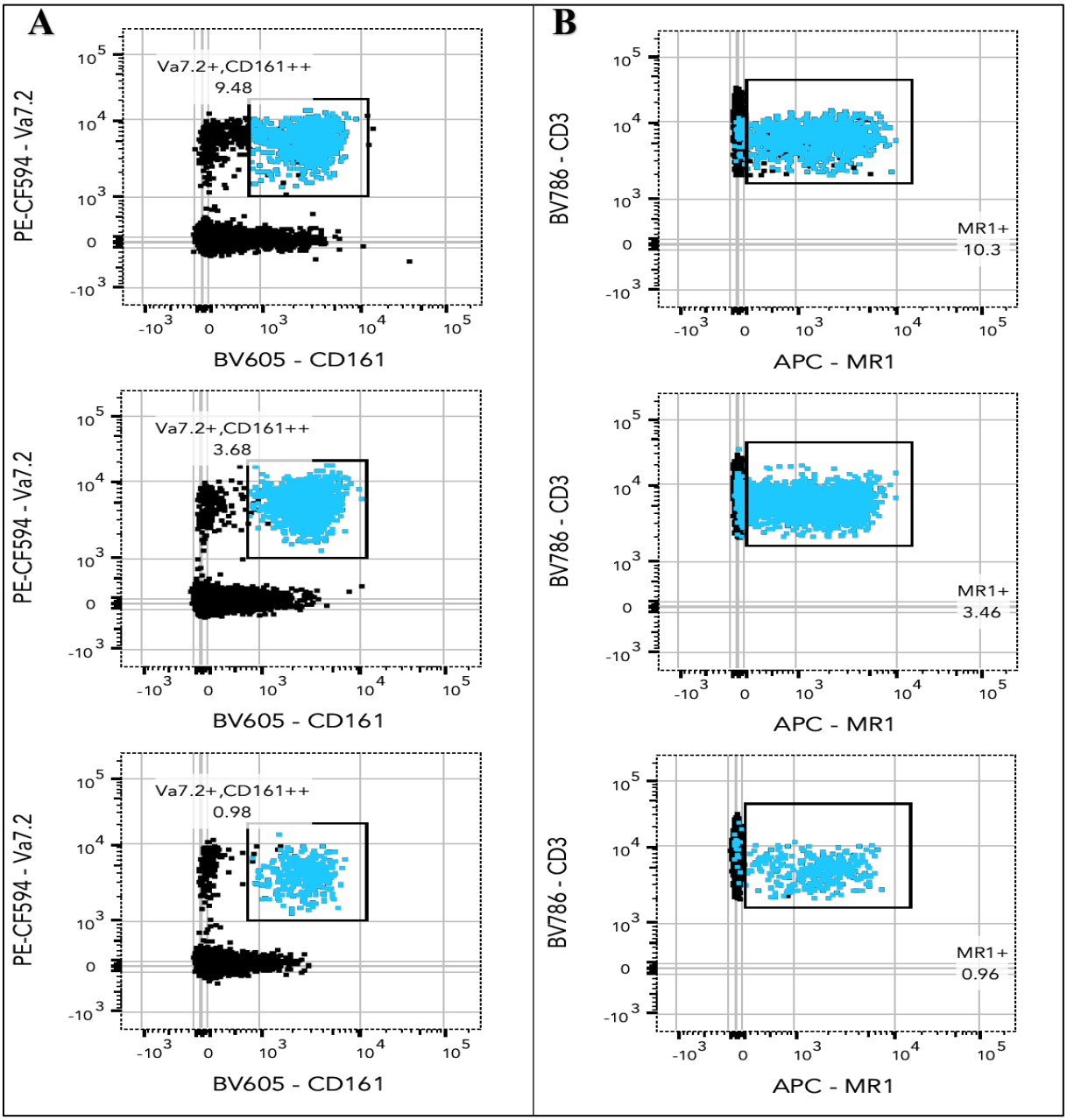


Figure 8. Comparison of MR1-5-OP-RU tetramer and Vα7.2$^+$, CD161^{hi} surrogate phenotyping. (A) Black dots represent MR1 tetramer$^-$ cells, blue dots represent MR1 tetramer$^+$ cells, and the gate represents the Vα7.2$^+$, CD161^{hi} cells from three healthy donors. MAIT cells identified with Vα7.2 CD161 were, on average 94.1% positive for MR1. (B) Black dots represent Vα7.2$^-$, CD161$^{-/low}$ cells, blue dots represent Vα7.2$^+$, CD161^{hi} cells, and the gate represent MR1 tetramer$^+$ cells from 3 healthy donors. MAIT cells identified defined by MR1$^+$ were, on average 93.2% positive for Vα7.2, CD161. The prior gating was performed as follows: Flow/Singlets/Lymphocytes/ Live/CD3$^+$ cells before plotting CD3 vs MR1 tetramer or Vα7.2 vs CD161.

First, we determined the proportion of MR1-5-OP-RU tetramer^{+} cells that are represented in the MAIT cell subsets, as defined by CD161^{hi} V**α**7.2^{+} (***Figure 8A***). In all three donors, a high proportion MR1-5-OP-RU tetramer^{+} was observed in the V**α**7.2^{+}, CD161^{hi} population, 86.8%, 97.2%, and 98.3%, respectively. Hence, it is clear that these markers highly enrich for MAIT cells. Yet, in line with the prior research, there exists a small proportion within this population that fail to label with the MR1-5-OP-RU tetramer. Furthermore, there also exists MAIT cells, classified by the recognition of MR1, that are not identified by the markers V**α**7.2 and CD161 in healthy individuals (***Figure 8B***). Normal inter-donor variability can be observed in both the percentage of MAIT cells from each donor, and the proportion in which MR1-5-OP-RU tetramer^{+} cells are co-labelled with V**α**7.2 and CD161. However, for the objectives of this project, and to simplify the experimental pipeline, we reasoned that the identification of MAIT cells using CD3^{+}, V**α**7.2^{+}, and CD161^{hi} would be sufficient.

6.2 Upregulation of Helios in MAIT cells upon CD3/CD28 stimulation

Prior research on the transcription factor Helios in T cells has predominantly been focused on regulatory T cells. Here, we evaluate the expression profile of Helios in MAIT cells, in order to validate the timepoint in which the upregulation peaks following CD3/CD28 stimulation. PBMCs from 3 healthy donors were freshly isolated and stimulated with CD3/CD28 before evaluating the expression level of Helios in T cells, specifically MAIT cells, in intervals over a period of 48 hours. In mucosal associated invariant T cells, identified on the basis of V**α**7.2^{+} and CD161^{hi}, a clear shift in Helios expression was observed following stimulation (***Figure 9C***). A significantly higher expression can be observed when compared to non-MAIT cells (V**α**7.2^{+}CD161^{-}). The third individual showed slightly different kinetics for Helios expression compared to the other two donors, as the Helios expression profile peaked at 36hs rather than 24hs (***Figure 9B***). A one-way ANOVA was performed on the median

fluorescence intensity (MFI) of Helios expression. No statistical significance could be observed, however, the 24hs time point showed the closest to significance results (***Figure 9A***). Taking into account that our sample group was small and that we have previously observed the same trend in Helios upregulation peaking at 24hs, we reasoned that 24hs following stimulation would be the optimal time to analyse the effect that reduced Helios expression may have on the phenotype of MAIT cells.

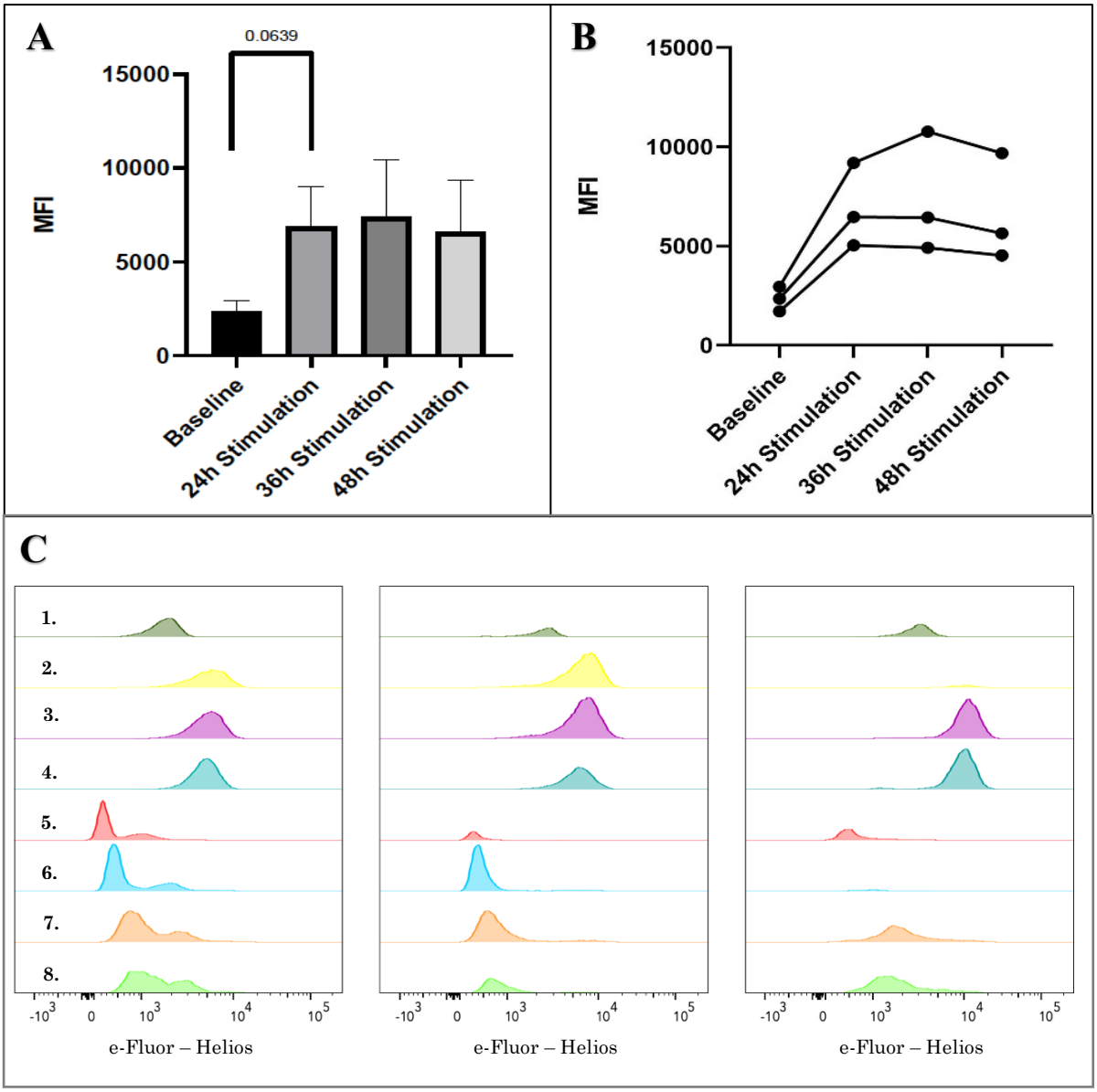


Figure 9. Upregulation of Helios following CD3/CD28 stimulation of MAIT cells in healthy donors. (A) Histogram representing Helios expression at baseline and 24 hs, 36 hs, and 48 hs after stimulation in independent biological triplicates. A two-way ANOVA analysis was performed. No statistical significance could be observed (B) Kinetics of Helios expression from each healthy donor (C) Comparison of the level of Helios expression between MAIT cells and non MAIT cells (Vα7.2^{+}, CD161^{-}) from each donor at all time points. 1-4. MAIT cells Helios expression at baseline, 24hs, 36hs, and 48hs following stimulation, respectively. 5-8. Non-MAIT cells Helios expression at baseline, 24hs, 36hs, and 48hs following stimulation, respectively. MAIT cells were identified by first gating for Flow/Singlets/Lymphocytes/ Live/CD3^{+}/Vα7.2^{+}CD161^{hi}.

The results from this experiment also provided information regarding the expression level of the TCR complex following CD3/CD28 stimulation. It became clear that following this type of stimulation, T cells downregulate their TCR complex considerably. The expression of both CD3 and V**α**7.2 was dramatically reduced when compared to unstimulated T cells (***Appendix***). Consequently, the identification of T cells and MAIT cells turn out to be more challenging with this type of stimulation. We attempted to circumvent this issue in the future experiment, where the accurate identification of these populations was required, by enriching for either CD3 or V**α**7.2 prior to CD3/CD28 stimulation.

6.3 Optimisation of Transfection Conditions

6.3.1 Transfection efficiency

To optimise the transfection efficiency of genetic material in unstimulated T cells, nucleofection, using two recommended settings from Lonza specifically developed for the transfection of unstimulated primary T cells, was utilised. One setting, FI-115 has been optimised for the high efficiency of delivery, while the other, EO-115, was developed for high functionality. Lonza also provides the media for the transfection that has also been optimised for the transfection of T cells. Thus, we evaluated the viability of T cells following the exposure to the transfection media and following mock transfection on either setting, comparing them to untreated T cells from the same donor (***Figure 10A***). Little difference could be observed in the viability of T cells exposed to the transfection media and the untreated T cells. However, as expected, the EO-115 and FI-115 mock transfected T cells showed a decrease in viability, due to the toxicity of the transfection itself, with the FI-115 transfection exhibiting the largest decrease; all measurements were taken at 24hs. To evaluate the potential disparity in transfection efficiency between the FI-115 and EO-115 settings, T cells were transfected with a GFP vector and cultured overnight before analysing the expression levels with flow cytometry (***Figure 10B***). The difference in

GFP expression that could be observed between the two settings was sizable. Therefore, despite the reduced viability of the T cells transfected with FI-115, the considerably higher transfection efficiency observed with this setting directed us to investigate whether a similar transfection efficiency could be observed in multiple donors, thus we repeated the GFP transfection in three donors with the FI-115 setting. (***Figure 10C***). Considerable variation between donors could be observed.

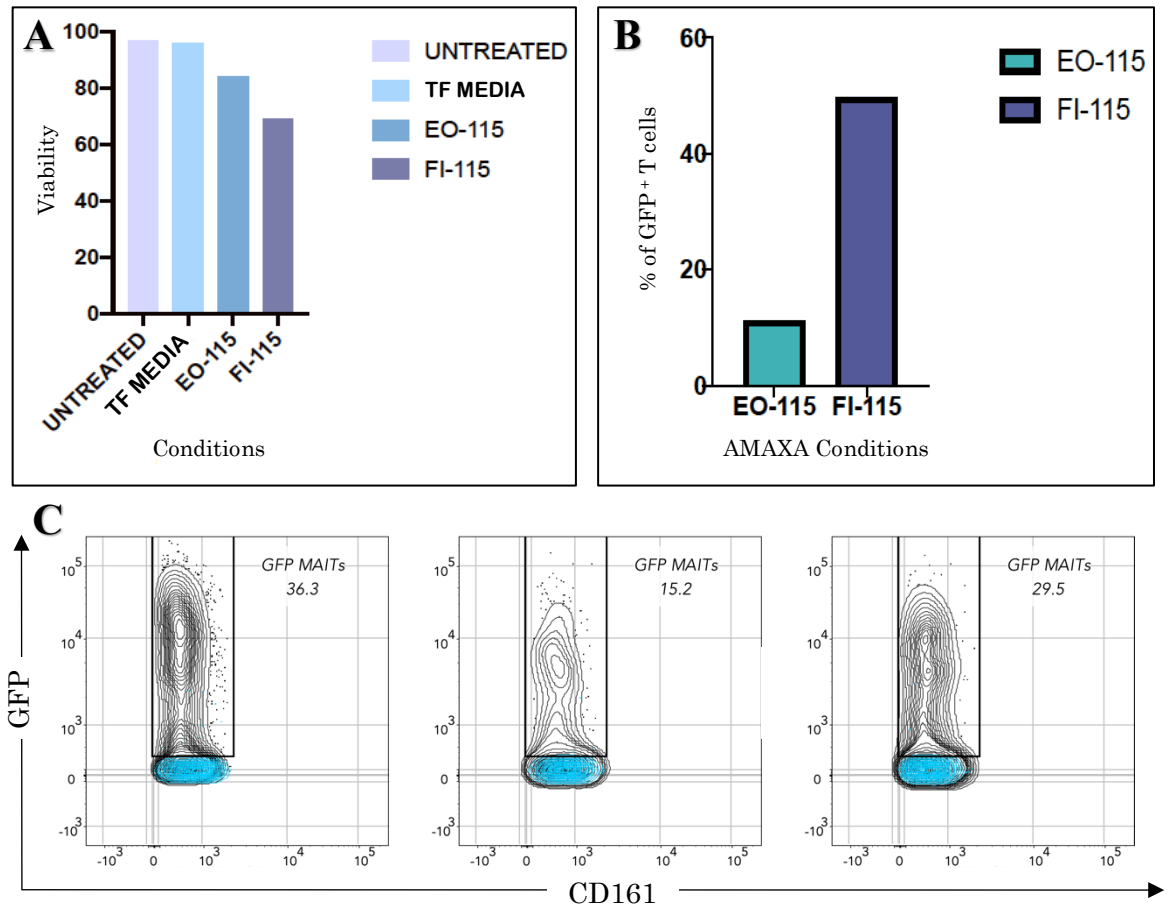


Figure 10. Optimisation of the transfection efficiency in unstimulated primary T cells. (A) Comparison of the viability between untreated T cells at 24hs and FI-115 or E0-115 mock transfected or following the exposure to the transfection (TF) media, in a single donor. (B) GFP expression in T cells, 24hs following transfection with pmaxGFP vector when transfected with either FI-115 (53.9%) or EO-115 (12.7%) setting, from a single donor. (C) GFP expression in MAIT cells from biological triplicates. The mock control from each donor (blue) was used to set the gates. T cells and MAIT cells were identified by first gating for Flow/Singlets/Lymphocyte/ Live/CD3^{+}/ and Vα7.2^{+}CD161^{hi}, respectively.

Next, we attempted to assess the functionality of the siRNA in primary T cells by utilising a positive control siRNA, which targets ubiquitously expressed human genes essential for cell survival. Knockdown of these genes should induce a high degree of cell death, whereas the negative control non-targeting siRNA is used to determine

the non-specific effects of siRNA delivery and provide the baseline to compare with siRNA-treated samples. Hence, when the viability of T cells transfected with the positive control siRNA is compared to T cells transfected with the negative control siRNA, the difference should represent the setting in which the functionality is the highest. Biological triplicates comparing the functionality of siRNA, when transfected with the different settings for unstimulated primary T cells, were performed and evaluated for viability 48hs post transfection. No significant difference between the positive control and negative control could be observed with either transfection setting (***Figure 11A***). The viability was also assessed at later time points, but still, no difference in viability between positive and negative control could be observed. Subsequently, it was evaluated whether the concentration of siRNA contributed to the lack of difference observed in the viability of the control siRNAs. A titration series of the positive control siRNA were evaluated for viability over a 48h period (***Figure 11B***). Here, no difference could be observed at either 24hs or 48hs between the different concentrations of positive siRNA and the negative control. However, it was clear that the prolonged time had a significant effect on the viability of the T cells transfected with siRNA, compared to the mock control.

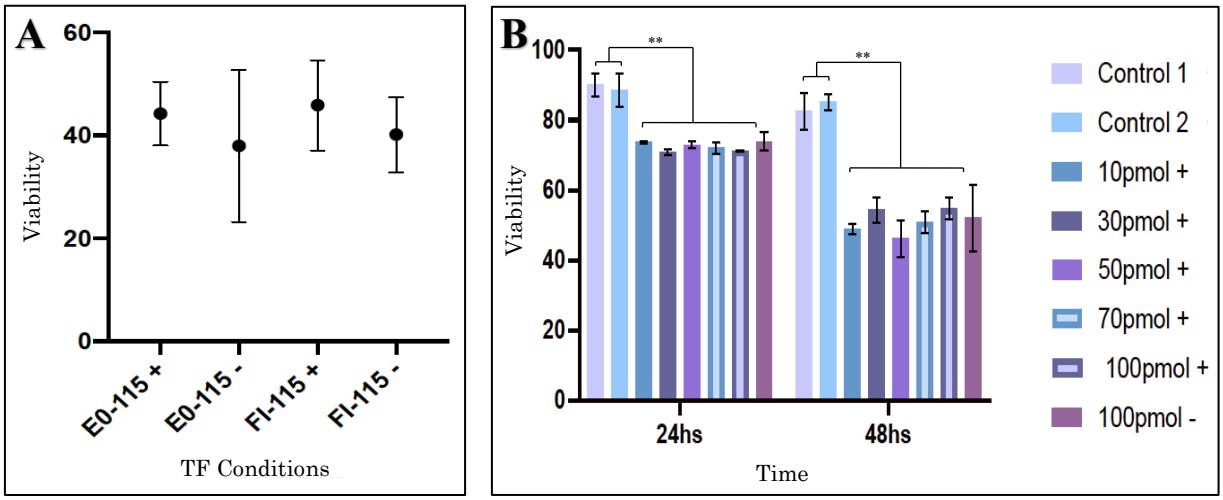


Figure 11. Optimisation of siRNA functionality. (A) The different settings for transfection of unstimulated T cells, FI-115 and EO-115, were evaluated for the functionality of the positive control siRNA (50pmol) based on the viability of T cells in biological triplicates (B) A series of concentrations of positive control siRNA were evaluated for viability at two different time points, 24- and 48- hours following transfection (FI-115). One-way ANOVA analysis. Control 1: Untreated T cells, Control 2: Transfection media treated T cells. ** = significance.

Due to the difficulty with the functionality of the positive control siRNA in primary T cells, siGLO, a fluorescently tagged siRNA, was used to investigate if the siRNA was able to enter T cells following the transfection, in the same manner as the GFP vector. Thus, it would act as a visual indication of siRNA delivery (***Figure 12***). Here, a slight shift in the FITC signal is visible following the transfection of siGLO in T cells. The siGLO signal was measured at 15, 48 and 72hs post transfection, and compared to the mock transfected control. The highest signal was present at 15hs, and over time the signal slightly decreased. At 15hs, a single sample was stimulated with CD3/CD28 to evaluate if the siRNA signal degenerates at an increased rate with stimulation, however, this did not seem to be the case (***Appendix***). Thus, we concluded that the siRNA was able to enter the T cells under the transfection conditions, and hence, the issues with the functionality of the positive control siRNA occurred for other reasons than entry, perhaps degradation of the siRNA, or incompatibility with primary T cells.

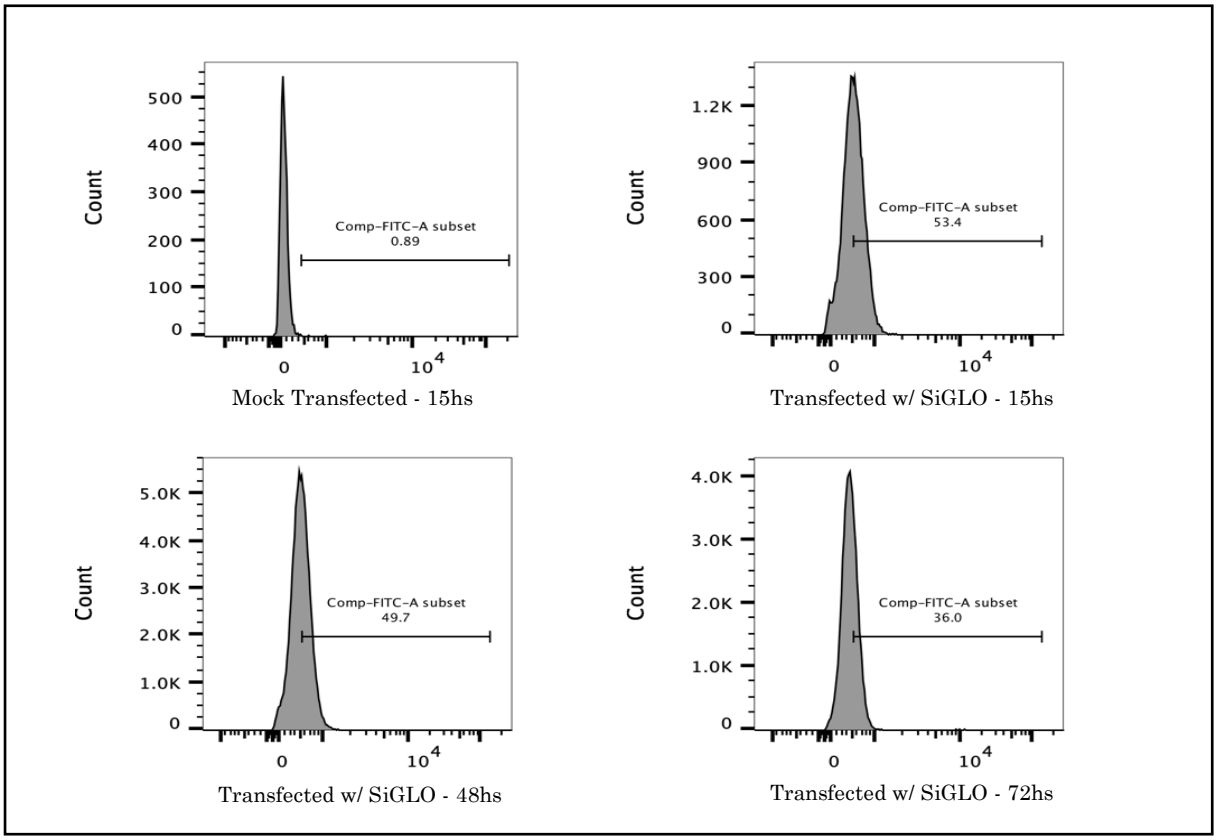
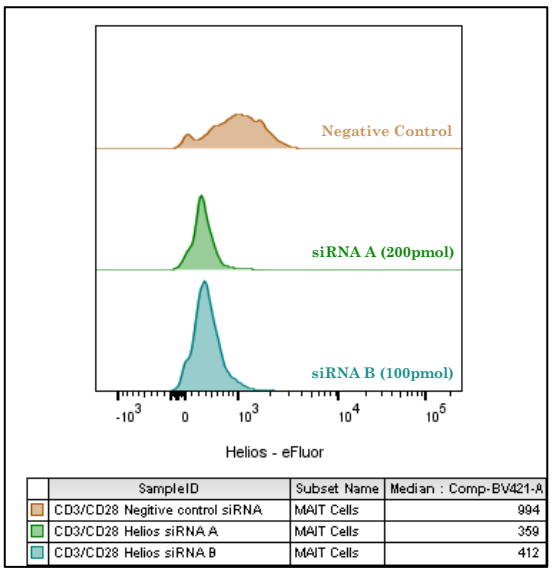


Figure 12. Transfection of T cells with siGLO. 100pmol of siGLO labelled with 6-FAM was transfected (FI-115) to unstimulated primary T cells of a single donor, and evaluated for signal at 15hs, 45hs and 72hs using flow cytometry. T cells were identified by first gating for Flow/Singlets/Lymphocytes/Live/$CD3^+$.

6.3.2 SiRNA Helios Knockdown

Despite the siRNA, that was used to serve as a positive control, not functioning as expected, we reasoned that the observation of sufficient levels of siRNA delivery from the siGLO transfected T cells warranted testing the functionality of the pooled Helios targeted siRNAs, using the FI-115 setting. Two concentrations (100pmol and 200pmol) of Helios targeted siRNA, were evaluated for their knockdown efficacy in MAIT cells, following 24hs of CD3/CD28 stimulation. The negative control siRNA was used as a baseline of Helios expression (***Figure 13***). A clear reduction in the Helios expression can be observed in both concentrations of Helios-targeted siRNA when compared to the negative control. The MFI of Helios in MAIT cells was evaluated to establish the percentage decrease in expression levels compared to baseline. The highest concentration of siRNA produced the largest knockdown effect at the protein level in this donor (63.9%), in contrast to the 100pmol concentration, which reduced Helios expression by 58.6%. Both concentrations of siRNA achieved the target of >50% that we aimed to reach. However, the higher concentration was selected for further experiments, partly due to the fact the transfection conditions could not be fully optimised and hence were perhaps suboptimal in multiple donors.



	SampleID	Subset Name	Median : Comp-BV421-A
	CD3/CD28 Negitive control siRNA	MAIT Cells	994
	CD3/CD28 Helios siRNA A	MAIT Cells	359
	CD3/CD28 Helios siRNA B	MAIT Cells	412

Figure 13. Optimisation of the siRNA concentration targeting Helios. Flow cytometric analysis representing the expression of Helios in MAIT cells transfected with 200pmol of negative control siRNA (baseline) or two concentrations (100 or 200pmol) of pooled Helios targeted siRNA following 24h CD3/CD28 stimulation in a single donor. MAIT cells were identified by first gating for Flow/Singlets/Lymphocytes /Live/CD3^{+}/ Vα7.2^{+} CD161^{hi}.

6.4 Evaluation of MAIT Cells Phenotype Following Knockdown of Helios.

Three buffy coats were obtained from the Finnish Blood service. The blood was donated one day (a maximum of 24hs) prior to the collection and processing of the buffy coat. Following the PBMC isolation and Vα7.2 enrichment, 200,000 cells from each donor were stained (***Table 3***) to evaluate the enrichment efficiency (***Figure 14A***). Both Donor 1 and 2 showed an extremely high Vα7.2^{+} enrichment efficiency (>95% of live CD3^{+}), Donor 3 however had a slightly lower efficiency.

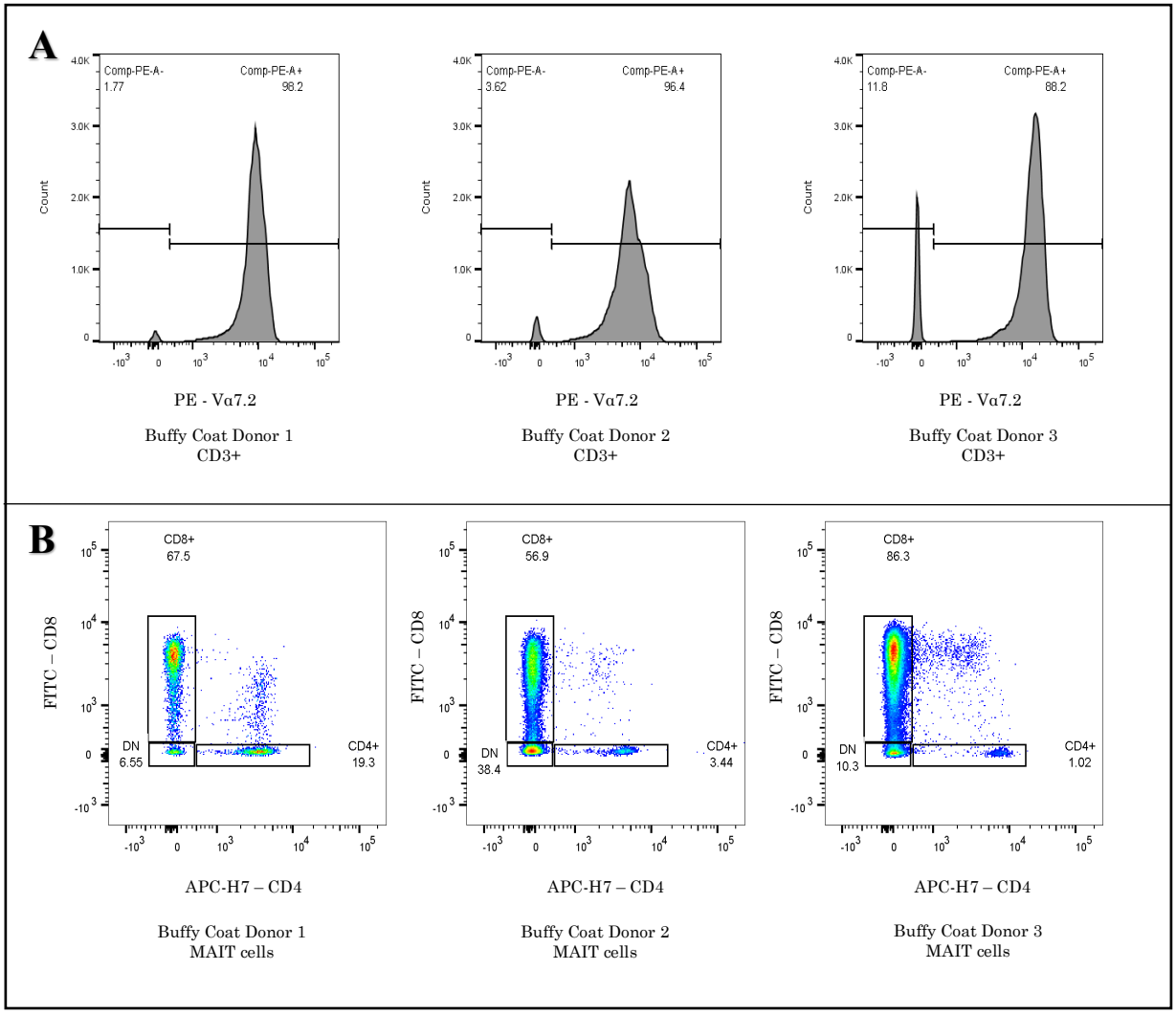


Figure 14. Efficiency of Vα7.2 enrichment and MAIT cell phenotype from the Buffy Coats of three donors. (A) Flow cytometric analysis of PBMCs enriched for Vα7.2. 98.2%, 96.4% and 88.2% of resulting T cells from each donor expressing Vα7.2, respectively. Gating for T cells was performed as follows; Flow/Singlets/Live/CD3+ (B) CD4/CD8 phenotype of each donors MAIT cells. Majority of the MAIT cell pool is CD8+, however, clear variation can be observed between donors DN and CD4+ MAIT subpopulations. The gating strategy for MAIT cells identification was implemented as follows: Flow/Singlets/Lymphocytes/Live/CD3^{+}/Vα7.2^{+}CD161^{hi}.

CD8 was unable to be included in the final panel (***Table 5***). Therefore, the proportion of $CD4^+$, $CD8^+$ and DN MAIT cells from each donor were ascertained in this panel, in order to offer a more complete picture of the variation present within the MAIT cell population between donors (***Figure 14B***).

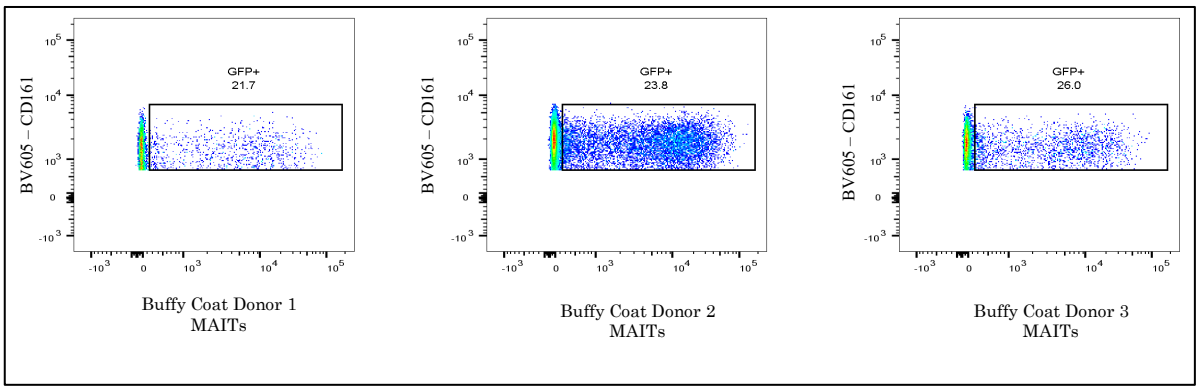


Figure 15. GFP expression of transfected MAIT cells from 3 Buffy Coat donors. Flow cytometric analysis of enriched Vα7.2 cells from each buffy coat donor which were transfected with GFP vector to provide a visual representation transfection efficiency. The expression level ranged between 21-26%. The gating strategy for MAIT cells identification was implemented as follows: Flow/Singlets/Lymphocytes/Live/$CD3^+$/$V\alpha7.2^+$, $CD161^{hi}$.

As a visual indication of delivery and the efficiency of transfection, GFP expression originating from the transfected vector, was evaluated. An internal control of delivery was not possible due to the fact the GFP signal dissipated upon fixation and permeabilisation. Thus, we included a separate sample as a proxy for the transfection efficiency, for which only the GFP vector was transfected (***Figure 15***). Between donors, the GFP expression ranged between 21-26% in MAIT cells. However, it is important to consider the size of a GFP vector is much larger than siRNA, and thus it is likely that the transfection efficiency of siRNA is much higher in reality.

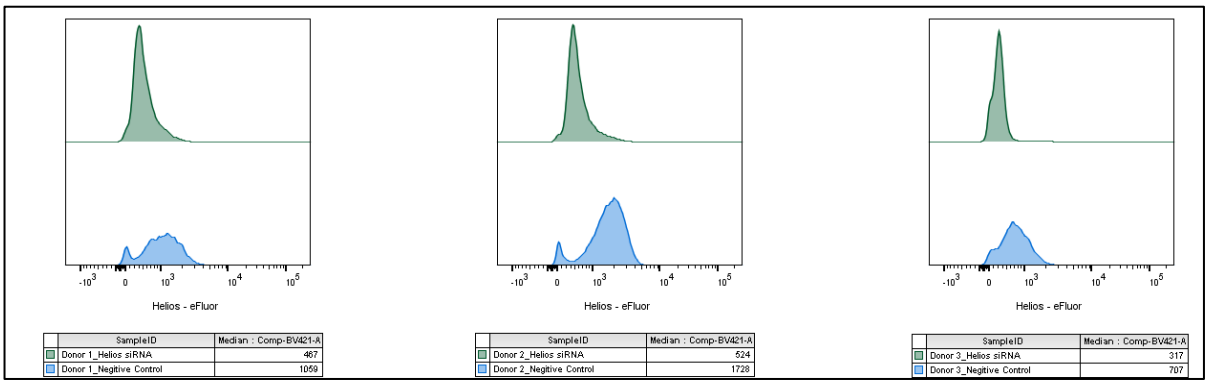


Figure16. The knockdown of Helios in MAIT cells from 3 Buffy Coat donors. Flow cytometric analysis representing the expression of Helios in MAIT cells transfected with a negative control (baseline) or 200pmol of pooled Helios targeted siRNA, following 24h CD3/CD28 stimulation for each buffy coat donor. Reduction when compared to negative control D1. 55.9%, D2. 69.7%, D3. 55.2%. The gating strategy for MAIT cells identification was implemented as follows: Flow/Singlets/Lymphocytes/Live/$CD3^+$/$V\alpha7.2^+CD161^{hi}$.

Following the transfection and the stimulation of the enriched Vα7.2^{+} T cells, the cells were stained with the MAIT cell phenotype panel (***Table 5***). Following the application of the gating strategy based on the FMO from each fluorochrome (excluding PE), MAIT cells were identified, and the Helios expression was evaluated to confirm the knockdown in each donor, compared to the mock and negative control. MFI values from all three donors indicated a higher than 50% knockdown, with an average of 60.3% reduction (***Figure 16***).

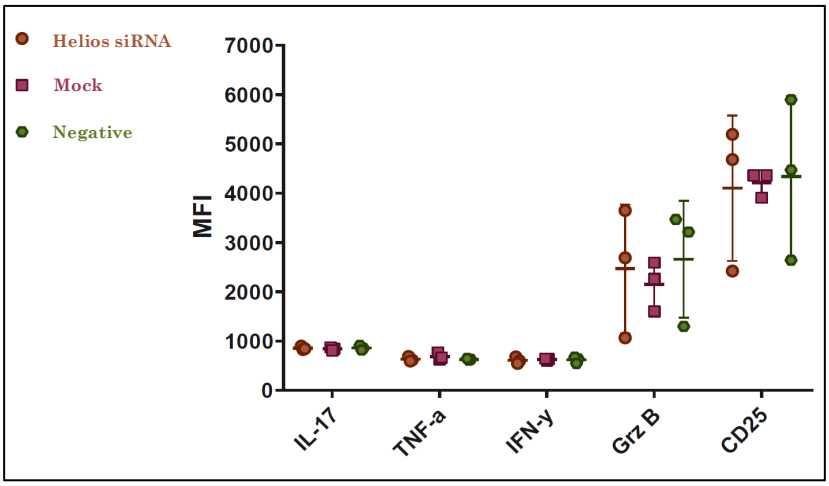


Figure 17. Evaluation of the activation markers of MAIT cells following the knockdown of Helios in MAIT cells from 3 Buffy Coat donors. Flow cytometric analysis was performed to evaluate changes in the expression level of the activation markers IL-17, TNFα, IFNγ, GrzB, CD25 between Helios knocked down MAIT cells and controls. No significant differences could be detected. The gating strategy for MAIT cells identification was implemented as follows: Flow/Singlets/ Lymphocytes/Live/CD3^{+}/Vα7.2^{+}CD161^{hi}/CD4^{-}.Gating for each marker was devised according to the corresponding FMO.

No significant differences could be detected in the expression levels between the Helios knocked down MAIT cells and the controls in any of the markers investigated (***Figure 17***). Ki-67, a marker of proliferation, showed no signal in any of the samples (***Appendix***). Moreover, few circulating MAIT cells are known to express IL-17, as this marker is typically expressed at higher levels in tissue resident MAIT cells[146]. This is in line with what we can observe here, with the knockdown in Helios showing no difference. Nevertheless, both TNFα and IFNγ, which are typically expressed highly in activated circulating MAIT cells, exhibited a much lower signal, from all of the donors MAIT cells that had been transfected, than what would be expected.

When we compared the expression level of the activation markers to non-transfected cells, however, it became evident that there was a clear difference in the level of soluble activation markers between mock transfected and non-transfected MAIT cells (***Figure 18***). This indicates that the functionality of MAIT cells following FI-115 transfection dramatically reduces the levels of effector molecules, specifically soluble cytokines, that are typically upregulated upon stimulation.

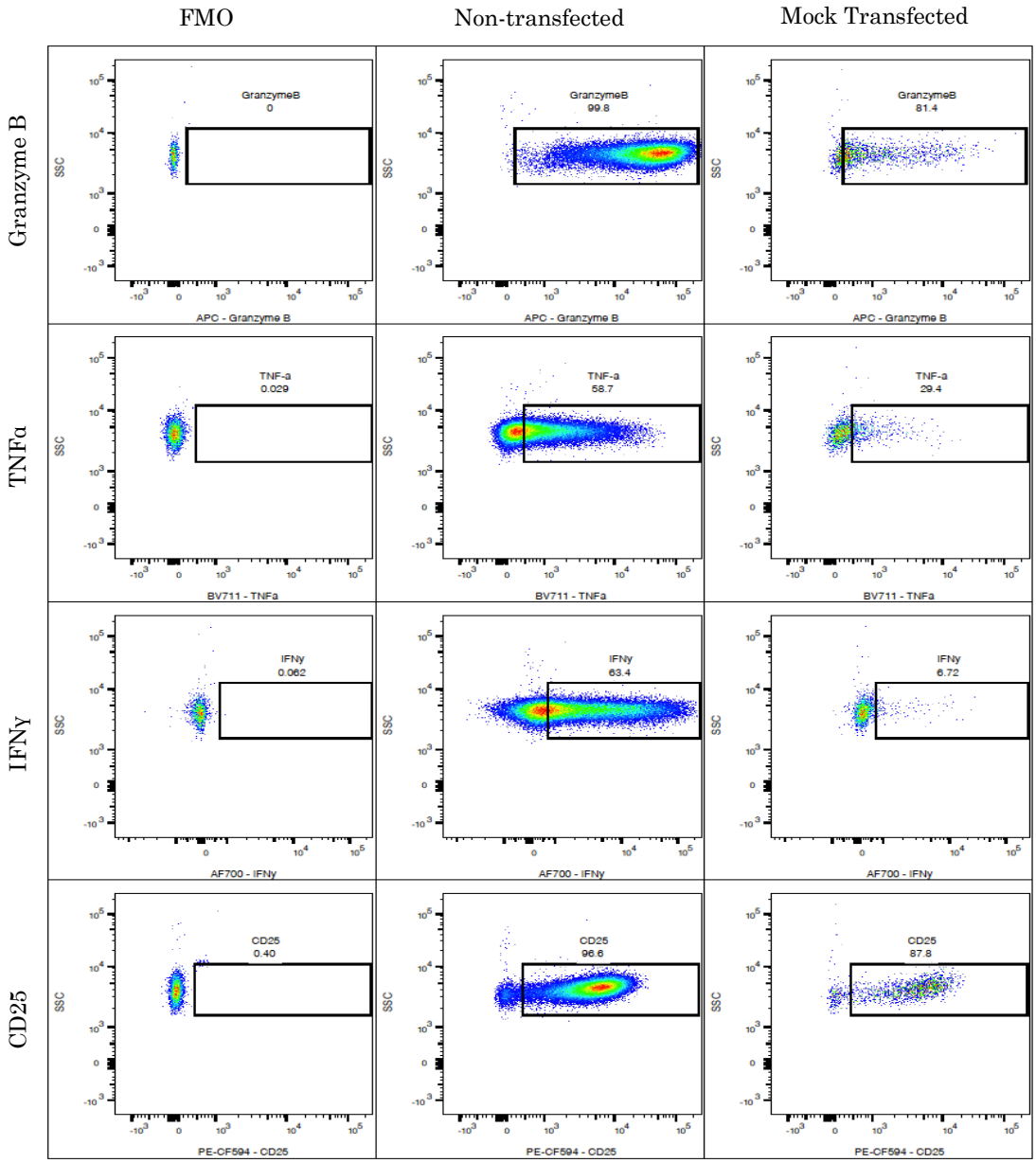


Figure 18. The functionality of MAIT Cells transfected with the high efficiency setting, FI-115. Flow cytometric analysis of MAIT cells from the third buffy coat donor was performed to analyse how the FI-115 transfection of MAIT cells impacted their functionality. Mock transfected MAIT cells (right column), which were stimulated with CD3/CD28 for 24hs, are compared to non-transfected stimulated MAIT cells (centre column) from the same donor. Gating for each marker was devised according to the corresponding FMO (left column). The gating strategy for MAIT cells identification was implemented as follows: Flow/Singlets/Lymphocytes/Live/$CD3^{+}$/$V\alpha7.2^{+}CD161^{hi}$ /$CD4^{-}$. Each marker was gated against SSC, and Ki-67 was excluded as no signal was observed from any sample.

7 DISCUSSION

The high frequency, potent antimicrobial activity and proinflammatory capacity of MAIT cells provide an appealing topic that is of great interest for both basic immunology and clinical research. Here, we aimed to increase our understanding of peripheral blood MAIT cells in healthy individuals by seeking to comprehend the role of Helios in their activation.

The identification of MAIT cells has typically been accomplished with flow cytometry by combining V**α**7.2 expression with high expression of CD161, however, since the development of the MR1-5-OP-RU loaded tetramer, which directly recognises MAIT cells by their MR1-restricted TCR specificity, this has become the most common and the gold standard of MAIT cell identification. Nevertheless, it has been acknowledged that in healthy donors, surrogate phenotyping techniques highly correspond to MAIT cells, defined by MR1 positivity[97]. Here, we evaluate how tightly these surrogate markers correlate with MR1 tetramer staining in donors that are considered to be healthy, and thus, whether it would be an appropriate method to identify MAIT cells for our experimental design. Our data confirmed what has been previously established, that $MR1^+$ cells highly correlate with V**α**7.2^+ $CD161^{hi}$ in healthy donors, yet, a small proportion of non-MAIT cells are falsely identified using these surrogate markers, and a minor subset of MAIT cells are excluded. Prior research has also shown that in situations where MAIT cell frequencies are reduced, such as with age or in disease settings, the proportion of non-MAIT cells in surrogate marker-defined populations increase[147].

Furthermore, it has been clearly demonstrated that the $CD8^+$ and DN V**α**7.2, CD161 subsets more closely represent the MAIT cell population than that of the CD4+ counterparts[81]. In one study, a population of T cells defined as $CD161^+$V**α**7.2^+CD4^+ exhibited a differential production of specific cytokines[148]. This could have resulted

from the fact that not all $CD4^+CD161^+V\alpha7.2^+$ T cells are MAIT cells, based on MR1 recognition. However, $CD4^+$ MAIT cells identified on the basis of MR1 recognition have also been shown to produce more IL-2 than other MAIT cell populations, and thus this population could potentially play a discrete role within the immune system[82]. Consequently, we reasoned that the $CD4^+Va7.2^+CD161^{hi}$ population should be excluded when evaluating the phenotypic functionality of MAIT cells.

The induced expression of Helios upon stimulation of the MAIT cell population emulates that of what has been observed in Tregs[149]. A rapid upregulation immediately following stimulation that peaks around 24hs. A similar expression pattern can also be observed upon bacterially derived stimulation, indicating that Helios plays an important role in the function and activation of MAIT cells[143]. In lymphocytes, Helios is known to act mainly as a transcriptional repressor. However, in contrast to Tregs, MAIT cells exhibit cytotoxic and effector functions, and thus, Helios has the potential to perform opposing roles within different T cell populations.

Moreover, earlier work performed in our laboratory observed an increase in proinflammatory signalling and production of proinflammatory cytokines in Helios haploinsufficient patients in a single family, signifying that the lack of Helios expression in T cells could cause heightened baseline levels of activation which thus leads to immune dysregulation. Additionally, one defining feature of these Helios haploinsufficient patients was the striking reduction in the number of MAIT cells in the peripheral blood, and the small number of $V\alpha7.2^+CD161^{hi}$ T cells that could be detected in patients exhibited lower levels of Helios expression than healthy controls. These findings led us to take a greater interest in the observation of proinflammatory activation markers of MAIT cells, such as IFNγ, TNFα, and Granzyme B, following the knockdown of Helios.

Due to the restricted number of markers that we could analyse simultaneously with flow cytometry, a clear limitation of our study, we were unable to evaluate all the markers that were of interest to us. Thus, we designed the panel to include what were believed to be the most relevant markers. Interestingly, however, it has been established that Helios represses IL-2 production in T cells[136]. Thus, the inclusion of IL-2 to the panel was of interest, but logistics made it difficult to include. Still, it is a marker that should certainly be evaluated in future studies, alongside markers such as Perforin, IL-7, IL-22 and IL-18R.

This study endeavoured to optimise a transfection method for the knockdown of Helios in MAIT cells. Preferably all the experiments executed to optimise the transfection conditions should have been performed in triplicates, however, due to the difficulties with the positive control siRNA, complete optimisations became challenging, and resources were directed towards solving the issue. The final condition utilised for the transfections were sub-optimal. However, a substantial knockdown in the expression of Helios at the protein level was achieved with a high concentration of Helios targeted siRNA. Nevertheless, lower concentrations of siRNAs are advised to avoid the heightened effects of off-targets[150].

In accordance with that, further experiments to optimise the transfection conditions for our experimental design should have been conducted. Primary T cells are difficult cells to transfect, and the impact of electroporation on T cell function, and not only viability, should have been considered. Hence, an evaluation of the functionality of the T cells following the transfection on the different settings, but prior to the addition of siRNA, would have been beneficial.

No significant differences could be detected in any of the markers between MAIT cells with reduced Helios levels and their controls. However, due to the high toxicity of the transfection conditions, we detected reduced functionality, which could explain

why we were unable to detect any differences in the expression levels of those markers. The soluble markers were particularly affected by the transfection, whereas the surface markers did not appear to be as impacted by the transfection. On the other hand, however, despite the considerable effects of the transfection on the functionality of the MAIT cells, it is also possible that Helios does not regulate any of the markers that we included in the panel, and comparable results may be observed with a gentler transfection setting and an equivalent knockdown effect.

One article describing MAIT cells from different age groups found that the percentages of IL-17-expressing cells in the circulating MAIT cells of healthy subjects were very low (around 1%−3%)[146]. These results are comparable with the data we observed, however, it is unreasonable to make any conclusions regarding the role Helios may or may not have on the expression of IL-17, or any of the other cytokines, due to the stunted expression of the soluble effector molecules following transfection. The expression levels of the CD25 however, were not so evidently impacted by the toxicity of the transfection, which theoretically could signify that Helios does not play any role in the upregulation of this marker upon activation in MAIT cells. However, further results would need to be ascertained to be able to make this conclusion. No Ki-67 expression was detected in any of the samples, however, due to the short stimulation and culture time of the experimental pipeline, it is not surprising, as proliferation in T cells typically occurs after 48hs in culture.

Therefore, from these results, it is difficult to conclude any clear role for Helios in the activation of MAIT cells, predominantly due to the fact that the transfection harshly affected the functionality of these cells, and consequently, our data is inconclusive.

All in all, there is still plenty of room for further optimisations of this experimental set up as well as further studies which could be performed to supplement these results and further validate the role of Helios in MAIT cells activation.

8 FUTURE RESEARCH

In this thesis, the experimental pipeline for the knockdown of the transcription factor Helios in primary MAIT cells was established. However, further optimisations are required to fully evaluate the effects reduced Helios expression has on the phenotype of MAIT cells during activation.

Research that would follow directly on from this project would be to establish the effects of a gentler transfection on the functionality of MAIT cells. This would subsequently require further optimisation of the concentration of siRNAs required to achieve a substantial knockdown effect in Helios. With this in mind, the evaluation of any potential off-target effects could be performed by assessing the phenotypic outcome following the transfection of each individual siRNA, rather than only the pooled siRNAs.

Once accomplished, and the experimental pipeline of this project has been repeated, the results of any phenotypic change of MAIT following the knockdown of Helios should be validated with a "closer to in vivo" type stimulation, a MAIT: Monocyte co-culture that can provide the opportunity for bacterially derived stimulation. Moreover, the ChIP-seq method could be used to analyse the interaction of Helios with DNA and identify its binding sites in MAIT cells.

Furthermore, an evident factor that limits the ability to fully comprehend the effect of the knockdown of Helios in MAIT cells is the restricted number of markers we can evaluate with flow cytometry. To evade this issue and to further validate our results, single-cell RNA sequencing could be utilised to gain a more precise understanding of the transcriptome in MAIT cells, with reduced Helios expression.

Despite the immense progress we have seen within the MAIT field in recent years, there is much research still to be done to fully understand the role of MAIT cells in the immune system.

9 REFERENCES

1. Sokol CL, Luster AD. The chemokine system in innate immunity. *Cold Spring Harb Perspect Biol*. 2015;7(5):a016303. Published 2015 Jan 29. doi:10.1101/cshperspect.a016303

2. Medzhitov R, Janeway CA Jr. Innate immunity: the virtues of a nonclonal system of recognition. *Cell*. 1997;91(3):295-298. doi:10.1016/s0092-8674(00)80412-2

3. Janeway CA Jr, Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002;20:197–216.

4. Coates M, Blanchard S, MacLeod AS. Innate antimicrobial immunity in the skin: A protective barrier against bacteria, viruses, and fungi. PLoS Pathog. 2018 Dec;14(12):e1007353.

5. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006 Feb 24;124(4):783–801.

6. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev. 2009 Apr;22(2):240–73, Table of Contents.

7. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. Front Immunol. 2014 Sep 25;5:461.

8. Borrego F. The first molecular basis of the "missing self" hypothesis. J Immunol. 2006 Nov 1;177(9):5759–60.

9. Ljunggren HG, Kärre K. In search of the "missing self": MHC molecules and NK cell recognition. Immunol Today. 1990 Jul;11(7):237–44.

10. Janeway CA Jr, Travers P, Walport M, Shlomchik MJ. Principles of innate and adaptive immunity. Garland Science; 2001.

11. Pradeu T, Jaeger S, Vivier E. The speed of change: towards a discontinuity theory of immunity?. *Nat Rev Immunol*. 2013;13(10):764-769. doi:10.1038/nri3521.

12. Comalada M, Peppelenbosch MP. Impaired innate immunity in Crohn's disease. Trends Mol Med. 2006 Sep;12(9):397–9.

13. Blach-Olszewska Z, Leszek J. Mechanisms of over-activated innate immune system regulation in autoimmune and neurodegenerative disorders. Neuropsychiatr Dis Treat. 2007 Jun;3(3):365–72.

14. Iwasaki A, Medzhitov R. Control of adaptive immunity by the innate immune system. Nat Immunol. 2015 Apr;16(4):343–53.

15. Jain A, Pasare C. Innate Control of Adaptive Immunity: Beyond the Three-Signal Paradigm. J Immunol. 2017 May 15;198(10):3791–800.

16. Krangel MS. Mechanics of T cell receptor gene rearrangement. Curr Opin Immunol. 2009 Apr;21(2):133–9.

17. Janeway CA Jr, Travers P, Walport M, Shlomchik MJ. T-cell receptor gene rearrangement. Garland Science; 2001.

18. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature. 1988 Aug 4;334(6181):395–402.

19. Williams RC. Regulation of Humoral Immunity. In: Jameson JL, editor. Principles of Molecular Medicine. Totowa, NJ: Humana Press; 1998. p. 251–8.

20. Apasov SG, Sitkovsky MV. T cell-mediated immunity. In: Nijkamp FP, Parnham MJ, editors. Principles of Immunopharmacology. Basel: Birkhäuser Basel; 2005. p. 19–28.

21. Janeway CA Jr, Travers P, Walport M, Shlomchik MJ. Immunological memory. Garland Science; 2001.

22. Kündig TM, Bachmann MF, Ohashi PS, Pircher H, Hengartner H, Zinkernagel RM. On T cell memory: arguments for antigen dependence. Immunol Rev. 1996 Apr;150:63–90.

23. Janeway CA Jr, Travers P, Walport M, Shlomchik MJ. T Cell-Mediated Immunity. Garland Science; 2001.

24. Kumar BV, Connors TJ, Farber DL. Human T Cell Development, Localization, and Function throughout Life. Immunity. 2018 Feb 20;48(2):202–13.

25. Fernández-Miguel G, Alarcón B, Iglesias A, Bluethmann H, Alvarez-Mon M, Sanz E, et al. Multivalent structure of an **αβ**T cell receptor. Proc Natl Acad Sci U S A. 1999 Feb 16;96(4):1547–52.

26. Roth DB. V(D)J Recombination: Mechanism, Errors, and Fidelity. Microbiol Spectr [Internet]. 2014 Dec;2(6).

27. Y. Sykulev, Y. Vugmeyster, A. Brunmark, H.L. Ploegh, H.N. EisenPeptide antagonism and T cell receptor interactions with peptide-MHC complexes. Immunity, 9 (1998), pp. 475-483.

28. S. Demotz, H.M. Grey, A. SetteThe minimal number of class II MHC-antigen complexes needed for T cell activation Science, 249 (1990), pp. 1028-1030.

29. Hennecke J, Wiley DC. T cell receptor-MHC interactions up close. Cell. 2001 Jan 12;104(1):1–4

30. Takahama Y. Journey through the thymus: stromal guides for T-cell development and selection. Nat Rev Immunol. 2006 Feb;6(2):127–35.

31. Fleury SG, Croteau G, Sékaly RP. CD4 and CD8 recognition of class II and class I molecules of the major histocompatibility complex. Semin Immunol. 1991 May;3(3):177–85.

32. Wucherpfennig KW, Gagnon E, Call MJ, Huseby ES, Call ME. Structural biology of the Tcell receptor: insights into receptor assembly, ligand recognition, and initiation of signaling. Cold Spring Harb Perspect Biol. 2010 Apr;2(4):a005140.

33. Germain RN. T-cell development and the CD4-CD8 lineage decision. Nat Rev Immunol. 2002 May;2(5):309–22.

34. Veillette A, Bookman MA, Horak EM, Bolen JB. Pillars article: the CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. 1994. J Immunol. 2010 Sep 1;185(5):2650–7.

35. Barabas AZ, Cole CD, Graeff RM, Lafreniere R, Weir DM. Tolerance, loss of tolerance and regaining tolerance to self by immune-mediated events. Immunol Res. 2017 Feb;65(1):402– 9.

36. Janeway CA Jr, Travers P, Walport M, Shlomchik MJ. Self-tolerance and its loss. Garland Science; 2001.

37. Palomares O, Yaman G, Azkur AK, Akkoc T, Akdis M, Akdis CA. Role of Treg in immune regulation of allergic diseases. Eur J Immunol. 2010 May;40(5):1232–40.

38. Gutcher I & Becher B. (2007) APC-derived cytokines and T cell polarization in autoimmune inflammation. J Clin Invest 117(5): 1119-1127.

39. Zhu J & Paul WE. (2008) CD4 T cells: fates, functions, and faults. Blood 112(5): 1557-1569.

40. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol. 1986 Apr 1;136(7):2348–57.

41. Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ ffector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol. 2005;6:1123-1132.

42. Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity*. (2009) 31:457–68. doi: 10.1016/j.immuni .2009.07.002.

43. Eyerich S, Eyerich K, Cavani A, Schmidt-Weber C. IL-17 and IL-22: siblings, not twins. Trends Immunol. 2010;31:354–61. doi: 10.1016/j.it.2010.06.004.

44. Albanesi C, Scarponi C, Cavani A, Federici M, Nasorri F, Girolomoni G. Interleukin-17 is produced by both Th1 and Th2 lymphocytes, and modulates interferon-gammaand interleukin-4-induced activation of human keratinocytes. J Invest Dermatol. 2000;115:81–7. doi: 10.1046/j.1523-1747.2000.00041.

45. Romagnani S. Human TH1 and TH2 subsets: regulation of differentiation and role in protection and immunopathology. Int Arch Allergy Immunol. 1992;98(4):279–85.

46. French, R. R., Chan, H. T., Tutt, A. L. and Glennie, M. J., CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help. *Nat. Med.* 1999. **5**: 548–553.

47. Commins SP, Borish L, Steinke JW. Immunologic messenger molecules: cytokines, interferons, and chemokines. J Allergy Clin Immunol. 2010 Feb;125(2 Suppl 2):S53–72.

48. Becskei A, Grusby MJ. Contribution of IL-12R mediated feedback loop to Th1 cell differentiation. FEBS Lett. 2007 Nov 13;581(27):5199–206.

49. Yamane H, Paul WE. Early signaling events that underlie fate decisions of naive CD4(+) T cells toward distinct T-helper cell subsets. Immunol Rev. 2013;252:12-23.

50. Zhu J. Transcriptional regulation of Th2 cell differentiation. Immunol Cell Biol. 2010;88:244-249.

51. Toellner KM, Luther SA, Sze DM, Choy RK, Taylor DR, MacLennan IC, et al. T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. J Exp Med. 1998 Apr 20;187(8):1193–204.

52. Kotsimbos AT, Hamid Q. IL-5 and IL-5 receptor in asthma. Mem Inst Oswaldo Cruz. 1997;92 Suppl 2:75–91.

53. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med. 1998 Jan 5;187(1):129–34.

54. Corry DB, Kheradmand F. Induction and regulation of the IgE response. Nature. 1999 Nov 25;402(6760 Suppl):B18–23.

55. Takeda K, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S, et al. Essential role of Stat6 in IL-4 signalling. Nature. 1996 Apr 18;380(6575):627–30.

56. Wurster AL, Tanaka T, Grusby MJ. The biology of Stat4 and Stat6. Oncogene. 2000 May 15;19(21):2577–84.

57. Ouyang W, Lohning M, Gao Z, et al. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. Immunity. 2006;12:27-37.

58. Zheng W, Flavell RA. 'e transcription factor GATA-3 is necessary and suffcient for Th2 cytokine gene expression in CD4 T cells. Cell. 1997;89:587-596

59. Manel N, Unutmaz D, Littman DR. 'e di$erentiation of human T(H)-17 cells requires transforming growth factorbeta and induction of the nuclear receptor RORgammat. Nat Immunol. 2008;9:641-649.

60. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity. 2006 Jun;24(6):677–88.

61. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006;441:235-238.

62. Song X, He X, Li X, Qian Y. The roles and functional mechanisms of interleukin-17 family cytokines in mucosal immunity. Cell Mol Immunol. 2016 Jul;13(4):418–31.

63. Aujla SJ, Chan YR, Zheng M, et al. IL-22 mediates mucosal host defense against gram-negative bacterial pneumonia. Nat Med. 2008;14:275-281.

64. Yasuda K, Takeuchi Y, Hirota K. The pathogenicity of Th17 cells in autoimmune diseases. Semin Immunopathol. 2019 May;41(3):283–97.

65. Eisenstein EM, Williams CB. The T(reg)/Th17 cell balance: a new paradigm for autoimmunity. Pediatr Res. 2009 May;65(5 Pt 2):26R – 31R.

66. Pereira CS, Macedo MF. CD1-Restricted T Cells at the Crossroad of Innate and Adaptive Immunity. J Immunol Res. 2016 Dec 14;2016:2876275.

67. Mori L, Lepore M, De Libero G. The Immunology of CD1- and MR1-Restricted T Cells. Annu Rev Immunol. 2016 May 20;34:479–510.

68. Sieling PA, Chatterjee D, Porcelli SA, et al. CD 1-restricted T cell recognition of microbial lipoglycan antigens. Science 1995;269:227–230.

69. Gherardin NA, Keller AN, Woolley RE, Le Nours J, Ritchie DS, Neeson PJ, et al. Diversity of T Cells Restricted by the MHC Class I-Related Molecule MR1 Facilitates Differential Antigen Recognition. Immunity. 2016 Jan 19;44(1):32–45.

70. Legut M, Cole DK, Sewell AK. The promise of **γδ** T cells and the **γδ** T cell receptor for cancer immunotherapy. Cell Mol Immunol. 2015 Nov;12(6):656–68.

71. Xiao X, Cai J. Mucosal-Associated Invariant T Cells: New Insights into Antigen Recognition and Activation. Front Immunol. 2017 Nov 10;8:1540.

72. Bertrand L, Lehuen A. MAIT cells in metabolic diseases. Mol Metab. 2019 Sep;27S:S114–21.

73. Smith DJ, Hill GR, Bell SC, Reid DW. Reduced mucosal associated invariant T-cells are associated with increased disease severity and Pseudomonas aeruginosa infection in cystic fibrosis. PLoS One. 2014 Oct 8;9(10):e109891.

74. Emgård J, Bergsten H, McCormick JK, Barrantes I, Skrede S, Sandberg JK, et al. MAIT Cells Are Major Contributors to the Cytokine Response in Group A Streptococcal Toxic Shock Syndrome. Proc Natl Acad Sci U S A. 2019 Dec 17;116(51):25923–31.

75. Koppejan H, Jansen DTSL, Hameetman M, Thomas R, Toes REM, van Gaalen FA. Altered composition and phenotype of mucosal-associated invariant T cells in early untreated rheumatoid arthritis. Arthritis Res Ther. 2019 Jan 5;21(1):3.

76. Porcelli S, Yockey CE, Brenner MB, & Balk SP (1993) Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. J Exp Med 178(1):1-16.

77. Tilloy F, Treiner E, Park S-H, Garcia C, Lemonnier F, de la Salle H, et al. An Invariant T Cell Receptor **α** Chain Defines a Novel TAP-independent Major Histocompatibility Complex Class Ib–restricted **α**/**β** T Cell Subpopulation in Mammals. J Exp Med. 1999 Jun 21;189(12):1907–21.

78. Treiner E, Duban L, Bahram S, Radosavljevic M, Wanner V, Tilloy F, et al. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. Nature. 2003 Mar 13;422(6928):164–9.

79. Lantz O, Bendelac A. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4–8— T cells in mice and humans. J Exp Med 1994;180:1097–1106.

80. Brozova J, Karlova I, Novak J. Analysis of the Phenotype and Function of the Subpopulations of Mucosal-Associated Invariant T Cells. Scand J Immunol 2016; 84: 245–251.

81. Dias J, Boulouis C, Gorin J-B, van den Biggelaar RHGA, Lal KG, Gibbs A, et al. The CD4-CD8- MAIT cell subpopulation is a functionally distinct subset developmentally related to the main CD8+ MAIT cell pool. Proc Natl Acad Sci U S A. 2018 Dec 4;115(49):E11513–22.

82. Gherardin NA, Souter MN, Koay H-F, Mangas KM, Seemann T, Stinear TP, et al. Human blood MAIT cell subsets defined using MR1 tetramers. Immunol Cell Biol. 2018 May;96(5):507–25.

83. Ussher J.E., Bilton M., Attwod E., Shadwell J., Richardson R., de Lara C., Mettke E., Kurioka A., Hansen T.H., Klenerman P., Willberg C.B. CD161++ CD8+ T cells, including the MAIT cell subset, are specifically activated by IL-12+IL-18 in a TCR-independent manner. Eur. J. Immunol. 2014;44:195–203.

84. Sharma PK, Wong EB, Napier RJ, Bishai WR, Ndung'u T, Kasprowicz VO, et al. High expression of CD26 accurately identifies human bacteria-reactive MR1-restricted MAIT cells. Immunology. 2015 Jul;145(3):443–53.

85. Dusseaux M, et al. (2011) Human MAIT cells are xenobiotic-resistant, tissuetargeted, CD161hi IL-17-secreting T cells. Blood 117(4):1250-1259.

86. Sato T, et al. (2004) Role for CXCR6 in Recruitment of Activated CD8+ Lymphocytes to Inflamed Liver. The Journal of Immunology 174(1):277-283.

87. Forster R, et al. (1999) CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell 99(1):23-33.

88. Wong EB, Gold MC, Meermeier EW, Xulu BZ, Khuzwayo S, Sullivan ZA, et al. TRAV1-2+ CD8+ T-cells including oligoconal expansions of MAIT cells are enriched in the airways in human tuberculosis. Commun Biol. 2019 Jun 5;2:203.

89. Kurioka A, et al. (2015) MAIT cells are licensed through granzyme exchange to kill bacterially sensitized targets. Mucosal Immunol 8(2):429-440.

90. Savage AK, et al. (2008) The transcription factor PLZF directs the effector program of the NKT cell lineage. Immunity 29(3):391-403.

91. Dias J, Sandberg JK, Leeansyah E. Extensive Phenotypic Analysis, Transcription Factor Profiling, and Effector Cytokine Production of Human MAIT Cells by Flow Cytometry. Methods Mol Biol. 2017;1514:241–56.

92. Leeansyah E, Svärd J, Dias J, Buggert M, Nyström J, Quigley MF, et al. Arming of MAIT Cell Cytolytic Antimicrobial Activity Is Induced by IL-7 and Defective in HIV-1 Infection. PLoS Pathog. 2015 Aug;11(8):e1005072.

93. Gérart S, Sibéril S, Martin E, Lenoir C, Aguilar C, Picard C, et al. Human iNKT and MAIT cells exhibit a PLZF-dependent proapoptotic propensity that is counterbalanced by XIAP. Blood. 2013 Jan 24;121(4):614–23.

94. Martin E, et al. (2009) Stepwise development of MAIT cells in mouse and human. PLoS Biol 7(3):e54.

95. Le Bourhis L, et al. (2010) Antimicrobial activity of mucosal-associated invariant T cells. Nat Immunol 11(8):701-708

96. Kjer-Nielsen L, Patel O, Corbett AJ, Le Nours J, Meehan B, Liu L, et al. MR1 presents microbial vitamin B metabolites to MAIT cells. Nature. 2012 Nov 29;491(7426):717–23.

97. Kurioka A, Jahun AS, Hannaway RF, Walker LJ, Fergusson JR, Sverremark-Ekström E, et al. Shared and Distinct Phenotypes and Functions of Human CD161++ V**α**7.2+ T Cell Subsets. Front Immunol. 2017 Aug 30;8:1031.

98. Gherardin NA, McCluskey J, Rossjohn J, Godfrey DI. The Diverse Family of MR1-Restricted T Cells. J Immunol. 2018 Nov 15;201(10):2862–71.

99. Chua WJ, et al. (2011) Endogenous MHC-related protein 1 is transiently expressed on the plasma membrane in a conformation that activates mucosal-associated invariant T cells. J Immunol 186(8):4744-4750.

100. Gold MC, et al. (2013) Human thymic MR1-restricted MAIT cells are innate pathogen-reactive effectors that adapt following thymic egress. Mucosal Immunol 6(1):35-44.

101. Seach N, et al. (2013) Double-positive thymocytes select mucosal-associated invariant T cells. J Immunol 191(12):6002-6009.

102. Koay HF, et al. (2016) A three-stage intrathymic development pathway for the mucosal-associated invariant T cell lineage. Nat Immunol 17(11):1300-1311.

103. Gold, M.C., Cerri S., Smyk-Pearson S., Cansler M.E., Vogt T.M., Delepine J., Winata E., Swarbrick G.M., Chua W.-J., Yu Y.Y.L., et al. . 2010. Human mucosal associated invariant T cells detect bacterially infected cells. PLoS Biol. 8:e1000407 10.1371/journal.pbio.1000407

104. Tastan C, Karhan E, Zhou W, Fleming E, Voigt AY, Yao X, et al. Tuning of human MAIT cell activation by commensal bacteria species and MR1-dependent T-cell presentation. Mucosal Immunol. 2018 Nov;11(6):1591–605.

105. Corbett AJ, Eckle SBG, Birkinshaw RW, Liu L, Patel O, Mahony J, et al. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. Nature. 2014 May 15;509(7500):361–5.

106. Keller AN, Eckle SBG, Xu W, Liu L, Hughes VA, Mak JYW, et al. Drugs and drug-like molecules can modulate the function of mucosal-associated invariant T cells. Nat Immunol. 2017 Apr;18(4):402–11.

107. Bosselut R, Ciucci T. Faculty Opinions recommendation of Drugs and drug-like molecules can modulate the function of mucosal-associated invariant T cells [Internet]. Faculty Opinions – Post-Publication Peer Review of the Biomedical Literature. 2017. Available from: http://dx.doi.org/10.3410/f.727280352.793530313'

108. Lezmi G, Leite-de-Moraes M. Invariant Natural Killer T and Mucosal-Associated Invariant T Cells in Asthmatic Patients [Internet]. Vol. 9, Frontiers in Immunology. 2018. Available from: http://dx.doi.org/10.3389/fimmu.2018.01766

109. Kurioka A, Ussher JE, Cosgrove C, Clough C, Fergusson JR, Smith K, et al. MAIT cells are licensed through granzyme exchange to kill bacterially sensitized targets. Mucosal Immunol. 2015 Mar;8(2):429–40.

110. Lamichhane R, Schneider M, de la Harpe SM, Harrop TWR, Hannaway RF, Dearden PK, et al. TCR- or Cytokine-Activated CD8+ Mucosal-Associated Invariant T Cells Are Rapid Polyfunctional Effectors That Can Coordinate Immune Responses. Cell Rep. 2019 Sep 17;28(12):3061–76.e5.

111. Salio M, Gasser O, Gonzalez-Lopez C, Martens A, Veerapen N, Gileadi U, et al. Activation of Human Mucosal-Associated Invariant T Cells Induces CD40L-Dependent Maturation of Monocyte-Derived and Primary Dendritic Cells. J Immunol. 2017 Oct 15;199(8):2631–8.

112. Salio M, Awad W, Veerapen N, Gonzalez-Lopez C, Kulicke C, Waithe D, et al. Ligand-dependent downregulation of MR1 cell surface expression. Proc Natl Acad Sci U S A. 2020 May 12;117(19):10465–75.

113. van Wilgenburg B, Scherwitzl I, Hutchinson EC, Leng T, Kurioka A, Kulicke C, et al. MAIT cells are activated during human viral infections. Nat Commun. 2016 Jun 23;7:11653.

114. Sugimoto C, Hirotani M, Yoshikiyo K, Koshimizu U, Wakao R, Horinouchi T, et al. The dynamics of mucosal-associated invariant T cells in multiple sclerosis. Springerplus. 2016 Aug 5;5(1):1259.

115. Carnero Contentti E, Farez MF, Correale J. Mucosal-Associated Invariant T Cell Features and TCR Repertoire Characteristics During the Course of Multiple Sclerosis. Front Immunol. 2019 Nov 20;10:2690.

116. Rouxel O, Da Silva J, Beaudoin L, Nel I, Tard C, Cagninacci L, et al. Cytotoxic and regulatory roles of mucosal-associated invariant T cells in type 1 diabetes. Nat Immunol. 2017 Dec;18(12):1321–31.

117. Chiba A, Tamura N, Yoshikiyo K, Murayama G, Kitagaichi M, Yamaji K, et al. Activation status of mucosal-associated invariant T cells reflects disease activity and pathology of systemic lupus erythematosus. Arthritis Res Ther. 2017 Mar 14;19(1):58.

118. Tominaga K, Yamagiwa S, Setsu T, Kimura N, Honda H, Kamimura H, et al. Possible involvement of mucosal-associated invariant T cells in the progression of inflammatory bowel diseases. Biomed Res. 2017;38(2):111–21.

119. Magalhaes I, Pingris K, Poitou C, Bessoles S, Venteclef N, Kiaf B, et al. Mucosal-associated invariant T cell alterations in obese and type 2 diabetic patients. J Clin Invest. 2015 Apr;125(4):1752–62.

120. Croxford JL, Miyake S, Huang Y-Y, Shimamura M, Yamamura T. Invariant V **α** 19i T cells regulate autoimmune inflammation. Nat Immunol. 2006;7(9):987–94.

121. Duan M, Goswami S, Shi J-Y, Wu L-J, Wang X-Y, Ma J-Q, et al. Activated and Exhausted MAIT Cells Foster Disease Progression and Indicate Poor Outcome in Hepatocellular Carcinoma. Clin Cancer Res. 2019 Jun 1;25(11):3304–16.

122. Gherardin NA, Loh L, Admojo L, Davenport AJ, Richardson K, Rogers A, et al. Enumeration, functional responses and cytotoxic capacity of MAIT cells in newly diagnosed and relapsed multiple myeloma. Sci Rep. 2018 Mar 7;8(1):4159.

123. Karamooz E, Harriff MJ, Lewinsohn DM. MR1-dependent antigen presentation. Semin Cell Dev Biol. 2018 Dec;84:58–64.

124. Georgopoulos K, Moore DD, Derfler B. Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. Science. 1992 Oct 30;258(5083):808–12.

125. John LB, Ward AC. The Ikaros gene family: transcriptional regulators of hematopoiesis and immunity. Mol Immunol. 2011 May;48(9-10):1272–8.

126. Rebollo A, Schmitt C. Ikaros, Aiolos and Helios: transcription regulators and lymphoid malignancies. Immunol Cell Biol. 2003 Jun;81(3):171–5.

127. Sridharan R, Smale ST. Predominant Interaction of Both Ikaros and Helios with the NuRD Complex in Immature Thymocytes [Internet]. Vol. 282, Journal of Biological Chemistry. 2007. p. 30227–38. Available from: http://dx.doi.org/10.1074/jbc.m702541200

128. Fan Y, Lu D. The Ikaros family of zinc-finger proteins. Acta Pharm Sin B. 2016 Nov;6(6):513–21.

129. Wang JH, Nichogiannopoulou A, Wu L, Sun L, Sharpe AH, Bigby M, et al. Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. Immunity. 1996 Dec;5(6):537–49.

130. Georgopoulos K, Bigby M, Wang JH, Molnar A, Wu P, Winandy S, et al. The Ikaros gene is required for the development of all lymphoid lineages. Cell. 1994 Oct 7;79(1):143–56.

131. Asanuma S, Yamagishi M, Kawanami K. Adult T-cell leukemia cells are characterized by abnormalities of Helios expression that promote T cell growth. Cancer [Internet]. 2013; Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/cas.12181

132. Georgopoulos K. The making of a lymphocyte: the choice among disparate cell fates and the IKAROS enigma. Genes Dev. 2017 Mar 1;31(5):439–50.

133. Getnet D, Grosso JF, Goldberg MV, Harris TJ, Yen H-R, Bruno TC, et al. A role for the transcription factor Helios in human CD4(+)CD25(+) regulatory T cells. Mol Immunol. 2010 Apr;47(7-8):1595–600.

134. Sebastian M, Lopez-Ocasio M, Metidji A, Rieder SA, Shevach EM, Thornton AM. Helios Controls a Limited Subset of Regulatory T Cell Functions. J Immunol. 2016 Jan 1;196(1):144–55.

135. Godfrey DI, Koay H-F, McCluskey J, Gherardin NA. The biology and functional importance of MAIT cells. Nat Immunol. 2019 Sep;20(9):1110–28.

136. Baine I, Basu S, Ames R, Sellers RS, Macian F. Helios induces epigenetic silencing of IL2 gene expression in regulatory T cells. J Immunol. 2013 Feb 1;190(3):1008–16.

137. Nakagawa H, Sido JM, Reyes EE, Kiers V, Cantor H, Kim H-J. Instability of Helios-deficient Tregs is associated with conversion to a T-effector phenotype and enhanced antitumor immunity. Proc Natl Acad Sci U S A. 2016 May 31;113(22):6248–53.

138. Himmel ME, MacDonald KG, Garcia RV, Steiner TS, Levings MK. Helios+ and Helios- cells coexist within the natural FOXP3+ T regulatory cell subset in humans. J Immunol. 2013 Mar 1;190(5):2001–8.

139. Cai Q, Dierich A, Oulad-Abdelghani M, Chan S, Kastner P. Helios deficiency has minimal impact on T cell development and function. J Immunol. 2009 Aug 15;183(4):2303–11.

140. Sebastian M, Lopez-Ocasio M, Metidji A, Rieder SA, Shevach EM, Thornton AM. Helios Controls a Limited Subset of Regulatory T Cell Functions. J Immunol. 2016 Jan 1;196(1):144–55.

141. Peters C, Oberg H-H, Kabelitz D, Wesch D. Phenotype and regulation of immunosuppressive V**δ**2-expressing **γδ** T cells. Cell Mol Life Sci. 2014 May;71(10):1943–60.

142. Serre K, Bénézech C, Desanti G, Bobat S, Toellner K-M, Bird R, et al. Helios is associated with CD4 T cells differentiating to T helper 2 and follicular

helper T cells in vivo independently of Foxp3 expression. PLoS One. 2011 Jun 3;6(6):e20731.

143. Miettinen S, Others. MAIT-solujen stimulaatio Escerichia coli ja Candida albicans mikrobeilla. 2018; Available from: https://helda.helsinki.fi/handle/10138/313170

144. Akimova T, Beier UH, Wang L, Levine MH, Hancock WW. Helios expression is a marker of T cell activation and proliferation. PLoS One. 2011 Aug 30;6(8):e24226.

145. Gibbs A, Leeansyah E, Introini A, Paquin-Proulx D, Hasselrot K, Andersson E, et al. MAIT cells reside in the female genital mucosa and are biased towards IL-17 and IL-22 production in response to bacterial stimulation. Mucosal Immunol. 2017 Jan;10(1):35–45.

146. Lu B, Liu M, Wang J, Fan H, Yang D, Zhang L, et al. IL-17 production by tissue-resident MAIT cells is locally induced in children with pneumonia. Mucosal Immunol. 2020 Sep;13(5):824–35.

147. Malka-Ruimy C, Ben Youssef G, Lambert M, Tourret M, Ghazarian L, Faye A, et al. Mucosal-Associated Invariant T Cell Levels Are Reduced in the Peripheral Blood and Lungs of Children With Active Pulmonary Tuberculosis. Front Immunol. 2019 Feb 14;10:206.

148. Garner LC, Klenerman P, Provine NM. Insights Into Mucosal-Associated Invariant T Cell Biology From Studies of Invariant Natural Killer T Cells. Front Immunol. 2018 Jun 28;9:1478.

149. Chougnet C, Hildeman D. Helios-controller of Treg stability and function. Transl Cancer Res. 2016 Aug;5(Suppl 2):S338–41.

150. Caffrey DR, Zhao J, Song Z, Schaffer ME, Haney SA, Subramanian RR, et al. siRNA off-target effects can be reduced at concentrations that match their individual potency. PLoS One. 2011 Jul 5;6(7):e21503.

10 ACKNOWLEDGEMENTS

Throughout the last two years, I have received a great deal of support and assistance, which has ultimately, led to the completion of this thesis. I have gained so much from so many, and words will never be able to fully describe the gratitude I feel, but here goes.

First and foremost, I would like to express my sincere gratitude to my research supervisor and PI, Dr. Eliisa Kekäläinen, for her continued guidance and support throughout this project. Your immense knowledge of immunology, enthusiasm for generating honest science, and "out the box" ideas, creates a captivating work environment, and I am very much looking forward to having you supervise my PhD studies.

Furthermore, I would also like to thank both Nelli and Iivo for sharing their expertise in flow cytometry with me, Iivari, for the time he spent teaching me in the lab, and the Arstila Group for sharing their space with us. A special thanks also goes out to our lab alumni, particularly, to Simo for guiding me through the continuation of his work. Thank you Joona and Suvi for your good-humoured presence in the lab; it is always a much better day when you guys are around. And to the rest of the Kekäläinen Lab, Adam, Pauliina, Shamita and Anh, thank you for being great colleagues to work with. And to our wider "research collective", it is great to be part of such a scientifically motivated team. Támas and Marjo, thank you for the technical support you provide to our lab; without you, we would all be lost. And to the Otonkoski Lab, thank you for kindly lending us the equipment that made this project possible.

Moreover, I would like to thank the entire Third Floor B Wing in Haartman, who always have encouraging words and friendly smiles, particularly, Liisa who made me feel at home, when I first arrived, with her kindness and infectious laugh. And to the rest of the kitchen crew, Larisa, Eva, Vinaya, Shimaa, Miguel, Marija, Usha, and Luz, thanks

for the amazing company and laughter, the coffee room feels a bit lonelier every time one of you leave. Lara, I couldn't be more thankful for your daily encouragement, you're the best! And to my fellow TransMed students, I will always be grateful that I was able to study alongside such sharp scientific minds, who motivated me to work harder and be better. I would specifically like to thank Chris, Ilse, Shivi, Marko, and Noora for enriching my life in Finland with all the fun and new experiences I have had along the way.

To my closest friends back at home, Emily, Jess, Tilly, Lucy and little Harrison, thank you for keeping me smiling and sane. The people in my life make getting through the tough times possible, and this is especially true of my family.

Otto, you have always been able to see potential in me, your loving support has encouraged me to achieve far beyond what I ever thought possible. For this, I will be forever indebted. You mean the world to me.

Mum, you are the person I look up to most, your strength, perseverance, and love for life have shown me what's important in life. I love you always. Dad, you taught me what it is to work hard, to reason well and think critically, thank you, and for always being there when I need you. Ultimately though, I would like to thank you both for the great genetics, and for two awesome siblings, Sam and Andy, who have undoubtedly taught me what it is to have people looking out for you.

Lastly, to my extended family Tom, Lena, Niki, Silke, Granddad, Ruth, Soheila and her beautiful family, and the ones no longer here, particularly Grandma Aideen, Janet, Arthur, and Anna, thank you for being part of my journey and supporting me every step of the way. I am incredibly lucky to have you all as my family.

11 APPENDIX

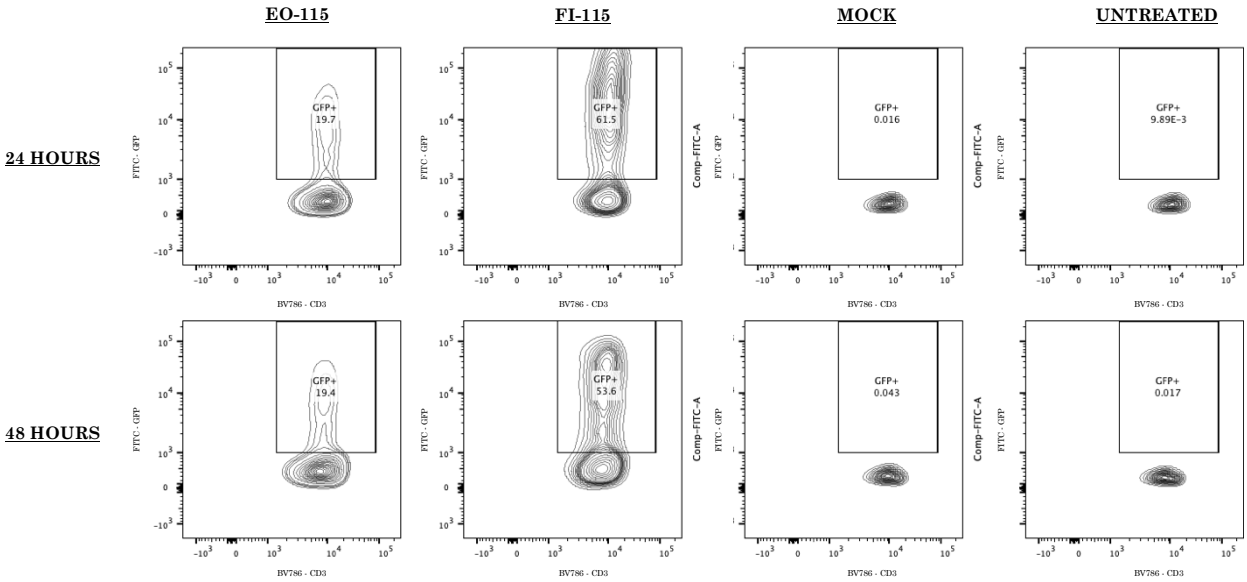


Figure 1. A Comparison of GFP expression at 24hs and 48hs following the transfection of a GFP vector in T cells. GFP expression in T cells of a single donor, 24hs or 48hs following transfection with pmaxGFP vector when transfected with either FI-115 or EO-115 setting. FI-115 shows a considerably higher expression level at both time points than the EO-115 setting. T cells were identified by gating for Flow/Singlets/Lymphocyte/ Live/CD3+.

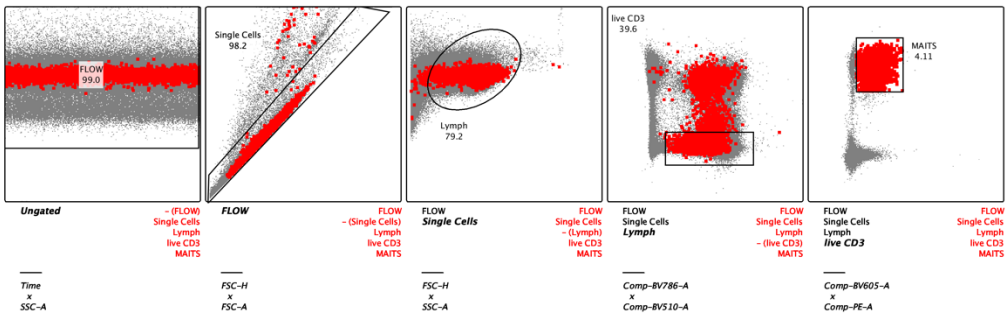


Figure 2. Representation of the Gating Strategy and Backgating utilised to Identify MAIT cells. Backgating is an advanced technique in flow cytometry data analysis, which illustrates a terminal gate of interest overlaid on previous points in the gating hierarchy without regard to that gate being illustrated. In settings where the BV605 – CD161 signal is low, and thus the identification of the MAIT cells becomes more difficult, backgating was applied to better refine the gating.

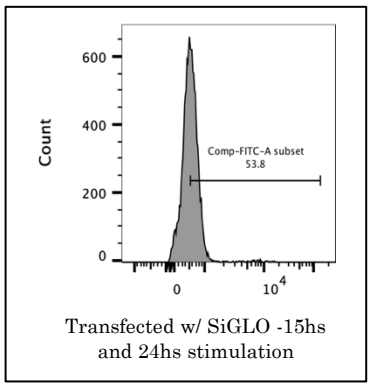


Figure 3. Transfection of T cells with siGLO and subsequent CD3/CD28 stimulation. 100pmol of siGLO labelled with 6-FAM was transfected (FI-115) to unstimulated primary T cells of a single donor, rested for 15hs and then stimulated for 24hs, before being evaluated for signal using flow cytometry. T cells were identified by first gating for Flow/Singlets/ Lymphocytes/Live/CD3+.

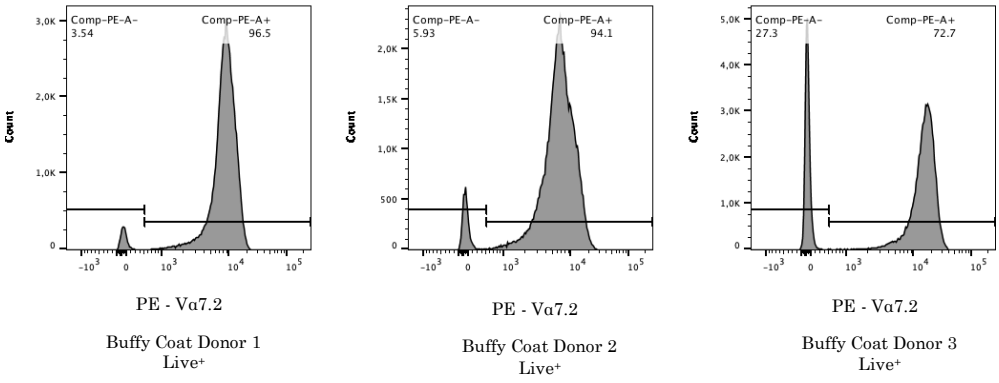


Figure 4. Efficiency of Vα7.2 Enrichment from Live PBMCs of Three Buffy Coats Donors. (A) Flow cytometric analysis of PBMCs enriched for Vα7.2. of the total Live cells from each donor expressing Vα7.2, respectively. Gating for T cells was performed as follows; Flow/Singlets/Live.

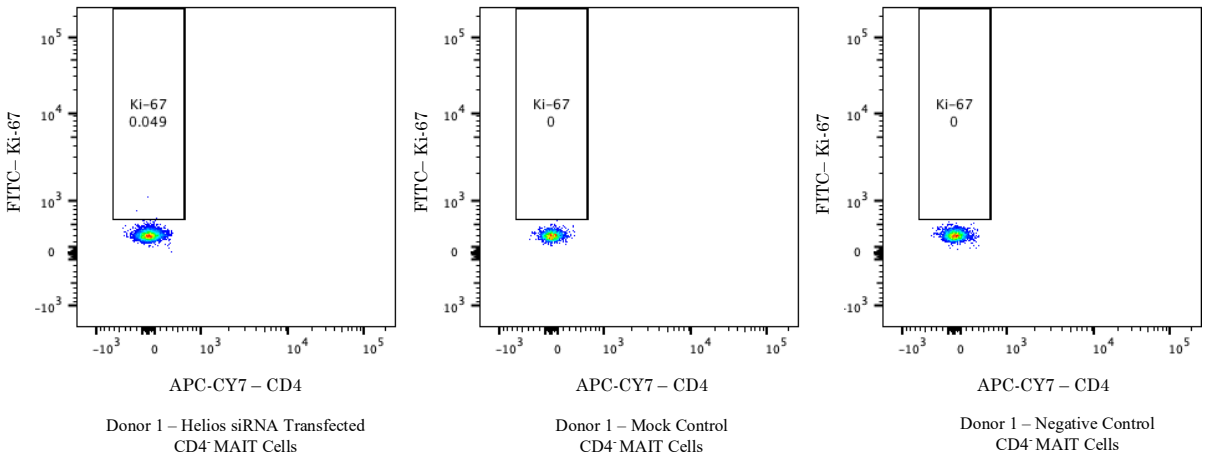


Figure 5. Representation of Ki-67 signal from the Buffy Coat Donors. Flow cytometric data of CD4- MAIT showing no signal from the proliferation marker KI-67. No change can be observed in the MAIT cells with a reduced expression of Helios. The gating strategy for MAIT cells identification was implemented as follows: Flow/Singlets/Lymphocytes/Live/CD3^{+}/Vα7.2^{+}CD161^{hi} /CD4^{-}.

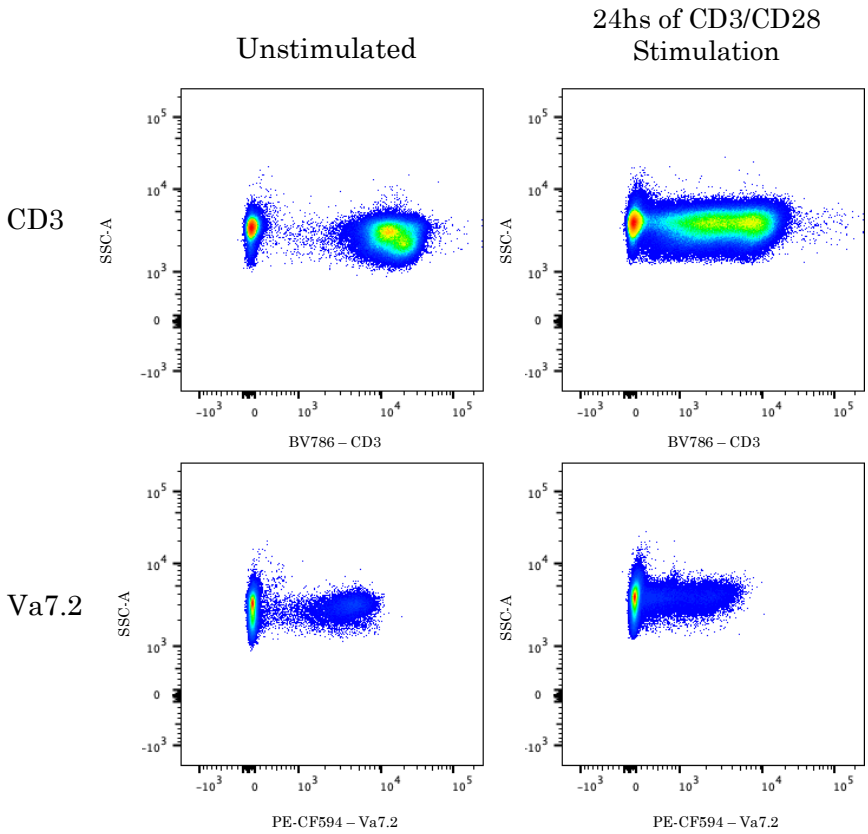


Figure 6. Representation of the Down regulation in CD3 and Vα7.2 upon CD3/CD28 Stimulation. Flow cytometric data showing that the markers, CD3 and Va7.2, in T cells are downregulated when stimulated with anti-CD3/CD28. The gating strategy for MAIT cells identification was implemented as follows: Flow/Singlets/Lymphocytes/Live/CD3^{+}/Vα7.2^{+}CD161^{hi}.